REMARKS

Prior to the present amendment, claims 1 to 20 were pending. Claim 58 has been added herein. Thus, claims 1 to 20 and 58 are pending and presently under examination.

Regarding the new claims

Claim 58, directed to the method of claim 1 in which the recited MEF2-encoding nucleic acid molecule is stably introduced into progenitor cells, has been added herein. New claim 58 is supported throughout the specification, for example, at page 55, lines 14-17, which indicates that permanent as well as transient expression can be useful in the methods of the invention, and at page 67, lines 9-21, which indicates that MEF2C was stably overexpressed in P19 cells. Thus, the new claim is supported in the specification as filed and does not add new matter. Accordingly, Applicants respectfully request that the Examiner enter new claim 58.

Regarding the rejections under the first paragraph of 35 U.S.C. § 112

The objection to the specification and corresponding rejection of claims 1 to 20 under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement are respectfully traversed.

The Office Action maintains the enablement rejection of the pending claims on two grounds. Essentially, the Office Action asserts that undue experimentation would have been required to (1) genetically modify human stem cells and (2) differentiate progenitor cells *in vivo*.

1. Regarding genetic modification of human stem cells

Claims 1 to 20 are directed to methods of differentiating progenitor cells including human progenitor cells such as human embryonic stem (ES) cells. The Office Action asserts that the specification fails to provide an enabling disclosure for genetic modification of human progenitor cells such as human ES cells, citing references published subsequent to the June 2000 priority date of the subject application. The publications cited by the Examiner allegedly report difficulties in transfecting human hematopoietic stem cells and report that transfection protocols established for mouse embryonic stem cells work poorly in human embryonic stem cells.

Applicants maintain that, in view of the specification, one skilled in the art would have been able to practice the claimed methods of differentiating progenitor cells without undue

experimentation. As argued previously, the specification teaches obtaining a variety of mammalian embryonic stem cells including stem cells from mice, cows, primates and humans, for example, human stem cells from infant cord blood or from adult peripheral blood (page 47, line 5, to page 51, line 12). As further guidance to the skilled person, the specification teaches methods for introducing a nucleic acid molecule into human or other progenitor cells (page 54, line 4, to page 55, line 17). As taught in the specification, well-known methods for introducing a nucleic acid molecule into progenitor cells such as embryonic stem cells include microinjection, electroporation, lipofection and viral-mediated techniques such as lentiviral and retroviral transduction (page 54, line 4, to page 55, line 17). Thus, the specification provides guidance to the skilled person regarding how to make and use the invention, including how to practice the invention with human progenitor cells.

As corroboration that one skilled in the art would have been able to introduce a nucleic acid molecule into human progenitor cells, Applicants submit herewith as Exhibit A Cheng et al., "Sustained Gene Expression in Retrovirally Transduced, Engrafting Human Hematopoietic Stem Cells and Their Lympho-Myeloid Progeny," Blood 92: 83-92 (1998). This article, which was published prior to the June 2000 priority date of the subject application, reports an optimized retroviral gene-transfer protocol clinically applicable to gene transfer into human hematopoietic stem cells (page 83, abstract). In particular, the 1998 paper by Cheng et al. reports the improved efficiency of their retroviral-mediated gene transfer protocol; as indicated at page 86, second column, greater than 30% of the CD34+/Lin- stem cells expressed the NGFR transgene (see, also, page 87, Figure 3). These result confirm the teachings of the specification indicating that retroviral vectors can be useful in the invention (see, for example, specification at page 55, lines 1-17). In sum, the 1998 publication by Cheng et al. provided as Exhibit A substantiates that one skilled in the art would have been able to introduce a nucleic acid molecule into human progenitor cells such human hematopoietic stem cells at the time the invention was made.

Furthermore, Applicants maintain that, as set forth in the specification, one skilled in the art would have been able to use other routine techniques such as electroporation to introduce a nucleic acid molecule into human or other progenitor cells. In this regard, the specification teaches that electroporation can be useful for introducing a nucleic acid molecule into embryonic stem cells in a method of the invention (page 54, lines 6-10) and that the invention can be

practiced with any of a variety of embryonic stem cells including human embryonic stem cells (page 28, lines 7-22). Applicants maintain that, irregardless of whether the methods of the invention have been reduced to practice using electroporation, the specification enables the invention by teaching that electroporation and other routine transfection methods can be useful in introducing a nucleic acid molecule into progenitor cells in a method of the invention.

Subsequent results also corroborate that electroporation would have been useful for introducing a nucleic acid molecule into human progenitor cells in accordance with the teachings in the specification. Applicants direct the Examiner's attention to the attached reference by Eiges et al. (Exhibit B), in which the efficiency of transfection into H9 human ES cells was assessed by determining luciferase reporter gene activity. As shown at page 515, Figure 1 of Eiges et al., luciferase reporter gene activity was observed in electroporated H9 human ES cells, indicating that these human progenitor cells were successfully transfected. Furthermore, Zwaka and Thomson, attached as Exhibit C, demonstrate that electroporation can be used to produce stably transfected human ES cells. At page 2, first sentence, Zwaka and Thompson report that electroporation of human ES cells yielded a stable transfection rate of 5.6×10^{-5}. See, also, page 1, second column, second full paragraph, of Zwaka and Thompson, which indicates that, following electroporation of 1.5×10^{7} human ES cells and selection with G418, 103 G418-resistant clones were obtained. Thus, subsequent results corroborate the teachings of the specification by demonstrating that only routine methods such as electroporation would have been required for the skilled person to practice the claimed invention with human progenitor cells.

Applicants maintain, as taught in the specification, that the claimed methods of differentiating progenitor cells can be practiced using either permanent or transient expression (specification at page 55, lines 14-17). For example, as is well known in the art and exemplified in the specification, antibiotic selection can be used to prepare progenitor cells in which a large percentage or all of the cells are selected to contain the introduced MEF2-encoding nucleic acid molecule. In this regard, the specification exemplifies stable transfection of P19 embryonic stem cells with MEF2C produced using selection with the antibiotic Geneticin (page 68, line 19, to page 69, line 4). Again, the specification provides guidance to the skilled person regarding the use of electroporation as well as permanent expression of MEF2-encoding nucleic acid

molecules. Subsequent work, as described above in Zwaka and Thompson, corroborates that electroporation can be used to prepare stably transfected human progenitor cells. Thus, as taught in the specification, stable as well as transient transfection can be useful in the claimed methods of differentiating progenitor cells.

Regarding the efficiency of transfection of human stem cells

The Office Action asserts that it is not clear what level of transfection efficiency is needed to carry out the claimed methods and further more, that it is not evident that low efficiency transfection would be sufficient for practicing the methods of the invention.

Applicants maintain that the claimed methods of differentiating progenitor cells do not require a particularly "high efficiency" of transfection of human or other progenitor cells. In particular, introducing a MEF2-encoding nucleic acid molecule into a small number of progenitor cells can be followed by antibiotic selection for the presence of the MEF2-encoding nucleic acid molecule. Thus, in combination with selection, low efficiency transfection of progenitor cells can be useful in the invention. Again, this point of view is corroborated by results of Zwaka and Thompson discussed above in which a transfection rate of about 5×10^{-5} was sufficient to produce more than 100 antibiotic-resistant colonies of human progenitor cells containing the transfected nucleic acid molecule. These results corroborate that, even if progenitor cells were not transfected with particularly high efficiency, one skilled in the art would have been able produce a population of progenitor cells predominantly or uniformly containing a MEF2-encoding nucleic acid molecule using routine methods.

2. *Regarding in vivo differentiation of embryonic stem cells*

The methods of the invention further stand rejected as allegedly lacking enablement for encompassing *in vivo* differentiation of progenitor cells. Applicants have previously argued that the presence of appropriate environmental cues in the *in vivo* neuronal environment promotes *in vivo* differentiation of progenitor cells containing a MEF2-encoding nucleic acid molecule. However, in refuting Applicants' position and dismissing positive results provided in supporting publications by Liu et al. and Deacon et al., the present Office Action asserts that the methods of the invention only have utility in diseased or injured animals and that the requisite "appropriate environmental cues" will not be present in such animals.

In contrast to the Examiner's assertion, it is well known in the art that growth and differentiation factors are present *in vivo* in the diseased or injured tissue environment. A number of factors with neurotrophic activies are known in the art including nerve growth factor (NGF), brain-derived growth factor (BDNF), ciliary neurotrophic factor (CNTF) and others (see, for example, Jacowski, attached as Exhibit D, paragraph spanning pages 308 and 309). Furthermore, the expression of growth and differentiation factors can be recapitulated or upregulated as compared to the expression of the factors in the normal adult nervous system. As an example, Jacowski report that peripheral nerve transection results in large quantities of NGR being produced by supporting Schwann cells and that central nervous system (CNS) injury "causes a time-dependent increase in neuronotrophic activity at a lesion site" (page 309, first column, second full paragraph). Thus, in contrast to the assertion in the Office Action, increased levels of nerve growth and differentiation factors are expected in diseased or injured tissue as compared to healthy tissue.

As additional evidence that appropriate environmental cures can be present in diseased or injured tissue, Applicants respectfully point out that the results of McDonald et al., "Transplanted Embryonic Stem Cells Survive, Differentiate and Promote Recovery in Injured Rat Spinal Cord," Nature Med. 5:1410-1412 (1999), previously submitted and provided herewith as Exhibit E, were achieved in mice subject to traumatic injury, specifically, impact injury of the spinal cord using a weight-drop device (page 1412, first column, second full paragraph). The results of McDonald et al. demonstrate that mouse embryonic stem cells survived and differentiated into astrocytes, oligodendrocytes and neurons following transplantation were achieved in acutely injured rather than healthy animals (page 1410, abstract, and second column).

Applicants' position also is supported by Liu et al., "Embryonic Stem Cells Differentiate into Oligodendrocytes and Myelinate in Culture and after Spinal Cord Transplantation," Proc. Natl. Acad. Sci. USA 97:6126-6131 (2000), previously submitted and provided herewith as Exhibit F. In particular, Liu et al. report embryonic stem cell transplantation experiments in two animal models: an "injury model" based on chemical demyelination and a "noninjury model" based on mice genetically deficient in myelin basic protein, emphasizing that the purpose of their studies was to determine whether embryonic stem cells can survive transplantation into both

injured and uninjured adult spinal cord (page 6126, second column, first complete paragraph; emphasis added). Liu et al. conclude that their studies demonstrate the ability of embryonic stem cell-derived oligodendrocytes to "replace lost myelin in the injured adult CNS (page 6126, abstract, penultimate sentence; emphasis added). Similarly, Liu et al. conclude that

> Our study demonstrates the ability of ES cell-derived oligodendrocytes to myelinate *in vitro* and to show that ES cells survive and myelinate axons in the mature and injured CNS after transplantation.... In particular, we demonstrate that injured, demyelinated areas of the adult CNS may preferentially stimulate oligodendrocyte differentiation/survival (page 6131, first paragraph).

These results confirm that differentiation can occur *in vivo* in animal models of nerve injury or disease. Applicants therefore respectfully submit that the Examiner's concerns regarding transplantation only being effective in healthy animals are not valid.

Regarding the cited publication by Jacowski

According to the Office Action, the cited publication by Jacowski allegedly supports the unpredictability associated with transplantation of neural tissue. Applicants respectfully disagree. At best, Jacowski discusses some difficulties associated with neural injury repair. However, Jacowski explicitly excludes foetal transplants from the difficulties seen in neural repair, reporting that embryonic cells can form axons when transplanted into the adult central nervous system (page 308, second full paragraph, "Foetal transplants seem exempt"). In view of Jacowski's favorable assessment of fetal transplants and transplanted embryonic cells, Applicants submit that one skilled in the art would have been able to practice the invention without undue experimentation.

Having addressed each of the Examiner's grounds for rejecting the claims as allegedly lacking enablement, Applicants request that the Examiner reconsider and remove the enablement rejection of claims 1 to 20 under 35 U.S.C. § 112, first paragraph.

CONCLUSION

Applicants respectfully request that the Examiner consider the amendments and remarks herein above. The Examiner is invited to call the undersigned agent or Cathryn Campbell if there are any questions relating to this application

To the extent necessary, a petition for an extension of time under 37 C.F.R. 1.136 is hereby made. Please charge any shortage in fees due in connection with the filing of this paper, including extension of time fees, to Deposit Account 502624 and please credit any excess fees to such deposit account.

Respectfully submitted,

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EXHIBIT A

Sustained Gene Expression in Retrovirally Transduced, Engrafting Human Hematopoietic Stem Cells and Their Lympho-Myeloid Progeny

By Linzhao Cheng, Changchun Du, Catherine Lavau, Shirley Chen, Jie Tong, Benjamin P. Chen, Roland Scollay, Robert G. Hawley, and Beth Hill

Inefficient retroviral-mediated gene transfer to human hematopoietic stem cells (HSC) and insufficient gene expression in progeny cells derived from transduced HSC are two major problems associated with HSC-based gene therapy. In this study we evaluated the ability of a murine stem cell virus (MSCV)-based retroviral vector carrying the low-affinity human nerve growth factor receptor (NGFR) gene as reporter to maintain gene expression in transduced human hematopoietic cells. CD34$^+$ cells lacking lineage differentiation markers (CD34$^+$Lin$^-$) isolated from human bone marrow and mobilized peripheral blood were transduced using an optimized clinically applicable protocol. Under the conditions used, greater than 75% of the CD34$^+$ cell population retained the Lin$^-$ phenotype after 4 days in culture and at least 30% of these expressed a high level of NGFR (NGFR$^+$) as assessed by fluorescence-activated cell sorter analysis. When these CD34$^+$Lin$^-$NGFR$^+$ cells sorted 2 days posttransduction were assayed in vitro in clonogenic and long-term stromal cultures, sustained reporter expression was observed in differentiated erythroid and myeloid cells derived from transduced progenitors, and in differentiated B-lineage cells after 6 weeks. Moreover, when these transduced CD34$^+$Lin$^-$NGFR$^+$ cells were used to repopulate human bone grafts implanted in severe combined immunodeficient mice, MSCV-directed NGFR expression could be detected on 37% ± 6% (n = 5) of the donor-type human cells recovered 9 weeks postinjection. These findings suggest potential utility of the MSCV retroviral vector in the development of effective therapies involving gene-modified HSC.

HEMATOPOIETIC STEM CELLS (HSC) provide an attractive target for somatic cell-based gene therapy because they have the potential to continue producing progeny cells containing a therapeutic gene indefinitely. Hematological diseases that could benefit from HSC-based gene therapy approaches include hereditary diseases as well as other diseases such as acquired immunodeficiency syndrome and cancer.[1] Retroviral vectors, which are being used in the majority of current clinical trials, are a primary choice as the vehicle for gene delivery. They are capable of integrating into cellular chromosomes, resulting in stable transmission to every progeny cell derived from transduced HSC.[2-4] However, it has become clear that current protocols for transducing human HSC with retroviral vectors are inefficient.[5,6] Although transgenes in engrafting cells have been detected using sensitive assays such as polymerase chain reaction (PCR), they have rarely been found in long-term repopulating cells.[5-7] These results are in striking contrast to the efficient transduction of less primitive human progenitors, which are able to form colonies under in vitro culture conditions,[7] and of mouse HSC.[8] Therefore, it is important to directly assess gene transduction into rare HSC capable of repopulating in vivo and of generating multiple (myeloid and lymphoid) lineages of differentiated hematopoietic cells.

Human HSC are included in a rare population of cells that bear the CD34 surface antigen (CD34$^+$) and lack all lineage differentiation markers (Lin$^-$). However, only a small fraction of CD34$^+$Lin$^-$ cells have HSC activities, operationally defined by long-term in vivo marrow repopulating activity and the ability to give rise to both myeloid and lymphoid progeny.[9,10] Several animal models have been developed in an attempt to detect HSC among the CD34$^+$Lin$^-$ population. One of these is the severe combined immunodeficient-human (SCID-hu) bone system, in which a human fetal bone fragment is implanted in SCID mice as a supportive human hematopoietic microenvironment.[11,12] Using this assay, it was found that the in vivo marrow repopulating activities of CD34$^+$Lin$^-$ cells mainly resided in a subset of cells expressing the Thy-1 antigen (Thy-1$^+$CD34$^+$Lin$^-$).[12,13] In contrast, Thy-1$^-$CD34$^+$Lin$^-$ cells were found to lack in vivo SCID-hu repopulating activity, although they were enriched for cells with in vitro colony-forming potential.[12,13] Analogous results have been obtained using other SCID mouse models and a human-fetal sheep model.[14,15] Thus, SCID repopulating cells (SRC) are considered to be more primitive than hematopoietic progenitor cells with in vitro activities, making the surrogate SCID-hu system a useful small animal assay to distinguish candidate human HSC from hematopoietic progenitors. In a recent report it was found that SRC within the CD34$^+$ population from human bone marrow (BM) were rarely transduced (<1%) even though retroviral-mediated gene transfer to colony-forming progenitors was highly efficient (up to 95% gene marking) under the conditions used.[14] The inability to efficiently transduce SRC in that study mirrors the low level of HSC transduction observed with existing protocols in various human gene therapy trials.[1,5-7]

The second potential problem associated with retroviral vector–based gene therapy is transcriptional silencing of the introduced transgene. Retroviral vectors derived from Moloney murine leukemia virus (MoMLV) are the most commonly used retroviral vectors in clinical trials.[2-4] In a standard configuration, the gene of interest is placed under the transcriptional control of the viral long terminal repeat (LTR) because gene expression driven by the LTR is generally higher than when the exogenous gene is under the control of an internal promoter.[16,17] However,

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it has been reported that MoMLV LTR-mediated gene expression is frequently downregulated during differentiation of HSC and inactive in several cell types.[17-19] Because the LTR of the murine stem cell virus (MSCV) retroviral vector is permissive for expression in murine HSC,[8,20] we were interested in examining the performance of the MSCV vector in candidate human HSC as well as in their differentiated progeny.

Recently, a number of groups have used various cell-surface molecules, including murine CD24 (HSA), murine CD8a (Lyt-2), and the low-affinity human nerve growth factor receptor (NGFR), to measure efficiency of gene transfer into hematopoietic precursors and to follow transgene expression in their marked progeny.[17,21,22] Retroviral vectors encoding NGFR have been used successfully by others to transduce human T lymphocytes and hematopoietic cells, and no adverse effects on the transduced cells have been observed.[22] Therefore, we decided to use the human NGFR gene as a selectable marker and reporter in this study. Multiparameter flow cytometric analysis allowed cell-surface expression of NGFR to be easily monitored in various hematopoietic cell populations defined by cell-surface markers (either $CD34^+Lin^-$ cell populations or differentiated cells belonging to a particular lineage). Similar efficiencies of gene transfer into colony-forming progenitors were obtained by a MSCV-based vector as for a MoMLV-based vector. However, we found that NGFR transgene expression mediated by MSCV LTR was substantially higher in differentiated erythroid, myeloid, and B-lymphoid progeny than that mediated by MoMLV LTR. Based on these findings we evaluated persistence of MSCV LTR-directed expression in progeny derived from transduced SRC. We show that our protocol for transducing SRC with the MSCV-based vector was efficient, and that a high percentage of transduced human cells continued to express NGFR after long-term reconstitution of SCID-hu bone mice.

MATERIALS AND METHODS

Construction and detection of retroviral expression vectors. The LXSN type of MoMLV vector was used as the parental vector for LINGFR.[23] After deleting the internal SV40 promoter and the *neo* gene in LXSN, an internal ribosome entry site (IRES) from the encephalomyocarditis virus was inserted.[24] The human (p75) NGFR gene was then placed after the IRES.[25] The resultant vector was named LINGFR to reflect the order of essential components (LTR-IRES-NGFR). The MINGFR vector was similarly constructed by replacing the *neo* gene driven by an internal promoter in MSCVneoEB with the IRES-NGFR cassette from LINGFR.[20] Gene expression mediated by the MINGFR vector is directed by the MSCV LTR, whereas in LINGFR gene expression is driven by the MoMLV LTR. A third vector, MINT, was created by truncating the NGFR gene in MINGFR after the transmembrane domain (at the *Nae* I site). All the plasmids were purified and used in packaging cell transfections as described.[26]

The primers used to amplify transgene-specific (IRES) DNA sequences common to all three vectors were: upstream, 5′-CGT TAC TGG CCG AAG CCG CT-3′; and downstream, 5′-AAC CTC GAC TAA ACA CAT GT-3′. The primers used to amplify the endogenous human β-globin sequence of genomic DNA as a control for PCR assays were: upstream, 5′-ACA CAA CTG TGT TCA CTA GC-3′; and downstream, 5′-CAA CTT CAT CCA CGT TCA CC-3′. Forty-cycle PCR reactions for both target sequences were performed with an annealing temperature of 62°C in the presence of 1.5 mmol/L $MgCl_2$. PCR products (a 485-bp IRES-specific fragment and a 220-bp β-globin–specific fragment) were separated by 4% agarose gel electrophoresis.

Specific antibodies for fluorescence-activated cell sorting (FACS) analyses. A hybridoma producing mouse IgG1 monoclonal antibody (MoAb) against the human NGFR was obtained from the American Type Culture Collection (ATCC HB8737; Rockville, MD). Purified antibodies from mouse ascites were conjugated either directly with fluorescein isothiocyanate (FITC) or with R-phycoerythrin (PE) after deleting the Fc fragment. A CD34 antibody (Tuk3) conjugated with sulfo-rhodamine (SR), and a panel of FITC-conjugated mouse MoAbs against lineage differentiation markers were used to isolate and analyze transduced cells.[27] This lineage panel (collectively called Lin) comprised CD2, CD4, CD14, CD15, CD16, CD19 (Becton Dickinson, San Jose, CA) and glycophorin A (Immunotech, Westbrook, ME). An FITC-conjugated antibody (MA2.1, ATCC HB54) against HLA-A2 was used to identify and monitor $MA2.1^+$ donor cells in SCID-hu bone mice.[12]

Propidium iodide (0.5 μg/mL) was added to cell suspensions after antibody staining to exclude dead/dying cells from FACS analyses. FACStar^{Plus} or FACS Vantage cell sorters (Becton Dickinson) equipped with a primary Agron laser and a dye-laser (required for detecting SR signals) were used for cell sorting. FACScan analyzers (Becton Dickinson) equipped only with an Argon laser were used to phenotype harvested cells after in vitro and in vivo assays.

Cytokines, media, and cell lines. Recombinant human interleukin-3 (IL-3), IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), steel factor (SLF, also called stem cell factor), and leukemia inhibitory factor (LIF) were obtained from Sandoz Pharma (Basel, Switzerland), and erythropoietin (Epo) was purchased from Amgen (Thousand Oaks, CA). Dulbecco's modified Eagle's medium (DMEM), Iscove's modified Dulbecco's medium (IMDM), and RPMI 1640 culture media were purchased from GIBCO-BRL (Gaithersburg, MD) and fetal calf serum (FCS) from Hyclone (Logan, UT). TF1 cells (ATCC CRL-2003) were maintained in RPMI 1640 plus 10% FCS and 2 ng/mL GM-CSF.[28]

Production of retroviral supernatants and transduction protocol. Amphotropic supernatants produced by the human 293 (embryonic kidney fibroblast) cell-based ProPak-A packaging line were made through transduction with VSV-G pseudotyped viral stocks as described.[29,30] NGFR-expressing ProPak-A cells were enriched by flow cytometry sorting and expanded in culture. Amphotropic supernatants were then collected from stable ProPak-A producers, filtered, and stored at −80°C until use. For transduction, fresh or previously frozen vector supernatants were mixed at a 1:1 ratio with media containing target cells in the presence of 8 μg/mL polybrene (Sigma, St Louis, MO). The transduction mixture was then centrifuged at 1,800*g* at 32°C to 35°C for inoculation. After the 4-hour "spinoculation," the cells were washed once and cultured in appropriate media.

Isolation and transduction of human hematopoietic progenitors. Human BM aspirates and mobilized peripheral blood (mPB, collected at day 4 or 5 after G-CSF treatment) were obtained from healthy donors in compliance with regulations established by the federal and state governments. Low-density (<1.077 g/cm^3) mononuclear BM cells after Ficoll-Hypaque gradient (Pharmacia, Piscataway, NJ) or apheresed mPB cells were stained with a CD34 antibody included in the Isolex kit (Baxter Biotech Immunotherapy Division, Irving, CA). $CD34^+$ cells were magnetically isolated by Isolex using a modified protocol developed at Systemix.[27] The purity of $CD34^+$ cells from both BM and mPB was usually greater than 90% (n = 10 for BM and n = 20 for mPB). These isolated cells were then stained with CD34-SR and Lin-FITC, and $CD34^+Lin^-$ cells were sorted by FACS and activated ex vivo for gene transduction. Cells (≈10^6/mL) were cultured overnight in IMDM/RPMI 1640 (1:1) medium plus 10% FCS supplemented with 10 ng/mL IL-3 and IL-6, and 100 ng/mL SLF. The next day cells were transduced with ProPak-A viral supernatants for 4 hours as described above. The transduction procedure was repeated the following day, and then the

medium was changed and the cells were cultured for additional 2 days to allow gene expression.

In vitro progenitor assays. Methylcellulose and reagents for clonogenic progenitor assays were obtained from StemCell Technologies (Vancouver, Canada). Cells (4×10^2) were added to 1 mL methylcellulose medium (MethoCult H4230) supplemented with IL-3, IL-6, GM-CSF (10 ng/mL each), SLF (100 ng/mL), and Epo (2 U/mL). Cell mixtures were plated in 35-mm suspension culture dishes (Nunc, Roskilde, Denmark), and incubated at 37°C. After 2 weeks, colonies (>100 cells) were enumerated in each of three triplicate plates. Subsequently some individual colonies were randomly picked. Cells were then lysed and an aliquot was used for a 40-cycle PCR analysis to detect specific DNA sequences. Colonies which yielded a positive signal for the human β-globin sequence were included in the calculations of colony-forming cell (CFC) gene transfer efficiency. Colonies which also yielded a transgene signal equal to or stronger than the β-globin signal in the same PCR reaction were considered to be positive for the transgene. The remaining cells were obtained in bulk at the end of 2-week CFC assays for FACS analysis of NGFR expression. Cells were washed three times with phosphate-buffered saline plus 0.5% human IgG (Gammimmune, Miles Inc, Elkhart, IN) before being stained with a PE-conjugated NGFR antibody and FITC-conjugated antibodies against glycophorin A, CD14, or CD15.

For stromal-dependent cobblestone area-forming cell (CAFC) assays,[31] a stromal cell line (SyS-1) derived from murine BM was used.[13] Cultures were maintained with IMDM/RPMI 1640 (1:1) medium plus 10% FCS supplemented with 10 ng/mL IL-6 and 50 ng/mL LIF. Up to 100 cells were cultured on sub-confluent monolayers of SyS-1 stromal cells in each well of 96-well plates. Half of the medium was replaced weekly, and the cultures were monitored for 6 weeks. CAFC frequencies were calculated based on the results obtained at week 5. Total cells were procured and pooled from wells which contained at least one cobblestone area at week 6. After being filtered through a 30-μm mesh filter, cells were stained as described before, with the PE-conjugated NGFR antibody and the FITC-conjugated CD19 antibody.

In vivo SCID-hu bone assays. Immunodeficient C.B-17 *scid/scid* (SCID) mice were used as recipients of human fetal bone fragments to construct the SCID-hu bone mice.[11,12] In each of two independent experiments, bone fragments were derived from the same human fetal tissue, which was negative for HLA-A2 and B17 and was not recognized by the corresponding MoAb MA2.1 (ATCC HB-54). Eight weeks after bone implantation, SCID-hu bone mice were used as recipients of transduced human hematopoietic precursors whose HLA-type was MA2.1$^+$. The transduced donor cells were injected directly into the implanted bones (MA2.1$^-$) residing in SCID mice according to the published protocol.[12] Fifty thousand to 100,000 cells (based on CD34$^+$Lin$^-$ cell counts) from the same cell population were injected into each of two bone fragments implanted in SCID mice. Nine weeks after injection, recipient mice were terminated, the implanted bone fragments were surgically removed, and total cells from BM were obtained. Then harvested cells were then stained with the PE-conjugated NGFR antibody and the FITC-conjugated MA2.1 antibody. Propidium iodide was added after antibody staining to cell suspensions to exclude dead/dying cells from FACS analyses. Using a FACScan analyzer, levels of engraftment (based on the presence of MA2.1$^+$ donor cells) and transgene expression (based on the presence of NGFR on the surface of donor cells) were assessed by FACS.

RESULTS

Transduction of human TF1 hematopoietic progenitor cell line with MSCV- and MoMLV-based vectors encoding NGFR. Several retroviral vectors containing the NGFR gene as a reporter were constructed to compare gene transfer and expression in human hematopoietic cells (Fig 1). NGFR expression is

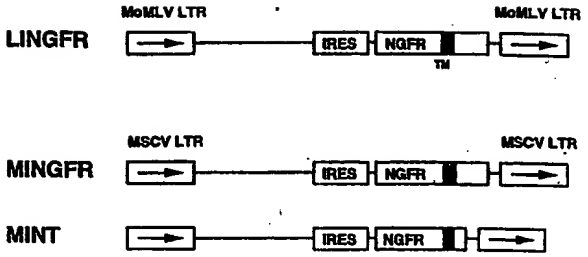


Fig 1. Schematic representation of NGFR-encoding retroviral vectors. The LXSN type of MoMLV vector was used as the parental vector backbone in the construction of LINGFR and the MSCVneoEB vector was used to derive the MINGFR and MINT vectors. In all cases, a NGFR gene has been placed downstream of an IRES, replacing the previous *neo* genes driven by internal promoters. Inclusion of the IRES potentially allows for coexpression of an upstream gene on bicistronic transcripts which also encode the NGFR reporter. The LTR of MoMLV directs NGFR gene transcription in LINGFR whereas the MSCV LTR is used to express the NGFR gene in MINGFR and MINT. The NGFR gene in MINT was truncated after the transmembrane (TM) domain.

directed by the MSCV LTR in MINGFR and MINT, or directed by the MoMLV LTR in the LXSN-derived vector LINGFR.[20,23] To achieve a higher efficiency of transduction of human hematopoietic precursors than has been routinely accomplished with recombinant retroviral stocks prepared using conventional murine packaging lines, amphotropic retroviral vector supernatants were produced using a new human 293 cell-based packaging line (ProPak-A).[29] Transduction efficiencies were first assessed on the growth factor–dependent human CD34$^+$ progenitor line TF1.[28] As shown in Fig 2A, ≈60% of TF1 cells were transduced by either MINGFR or LINGFR vector 4 days after the cells were exposed to 50% (vol/vol) of viral supernatants in a "spinoculation" protocol. Subsequent limiting dilution experiments with MINGFR and LINGFR vector preparations established that the two viral stocks had comparable titers (data not shown). Moreover, as indicated by the mean fluorescence intensities of the positive peaks, both vectors directed similar NGFR expression levels in TF1 cells (Fig 2A). The two vectors also performed equally well when tested on other cell lines such as murine NIH3T3 fibroblasts (data not shown).

A third vector, MINT, in which the NGFR gene in the MINGFR vector was C-terminally truncated after the transmembrane domain, was used to examine the kinetics of NGFR expression after viral transduction. The titer of MINT viral stock was somewhat lower and ≈40% TF1 cells were transduced and expressed NGFR on cell surface after 4 days (Fig 2A). To determine the kinetics of NGFR transgene expression after transduction, MINT-transduced TF1 cells were either stained for NGFR surface protein immediately posttransduction or cultured for various periods of time and then analyzed as described above. No NGFR signal could be detected on the cell surface immediately after the transduction protocol (Fig 2B). Subsequently, the percentage of NGFR-expressing (NGFR$^+$) cells as well as NGFR signal intensities progressively increased with time, reaching a maximum of 40% 4 days posttransduction (Fig 2A and B). Afterward the values of these two parameters remained constant for at least 2 months if transduced TF1 cells were maintained in a proliferating phase. The doubling time of

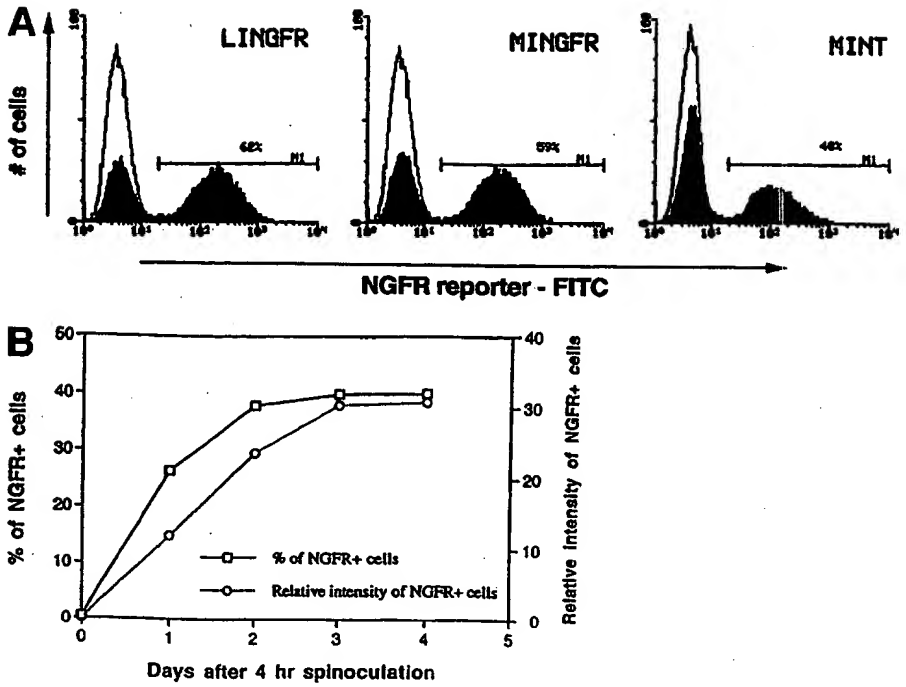


Fig 2. FACS analysis of NGFR expression in retrovirally transduced TF1 cells. (A) Histograms of TF1 cells expressing the NGFR transgene after transduction with the LINGFR, MINGFR, or MINT vectors. TF1 cells were transduced with equal volumes of amphotropic viral supernatants for 4 hours (as described in Materials and Methods) and NGFR expression was analyzed by FACS 4 days later after staining for cell-surface NGFR with a FITC-conjugated anti-NGFR antibody. The profile of nontransduced TF1 cells (thin lines representing 0.5%) was overlaid to highlight transduced cell NGFR$^+$ populations. Approximately 62%, 59%, and 40% of TF1 cells were transduced with the LINGFR, MINGFR, and MINT vectors, respectively. (B) Kinetics of NGFR expression in TF1 cells after transduction with the MINT vector. After 4-hour exposure to MINT vector supernatant, TF1 cells were either processed immediately (day 0) or cultured for 1, 2, 3, or 4 days and then stained for NGFR expression. Based on FACS histograms as shown in (A), the percentages of NGFR-expressing cells and relative levels of NGFR expression in transduced cells were plotted as a function of time in culture. The relative intensity of NGFR expression is defined by increased mean fluorescence intensity (MFI) normalized by the MFI of nontransduced cells.

TF1 cells is ≈20 hours; thus, it took a period equivalent to three cell divisions for continuously proliferating TF1 cells to reach the maximal and stable level of the NGFR surface signal. This finding is consistent with accumulated data that efficient retroviral-mediated expression (transcription and translation) occurs only after integration into cellular chromosomes which, in the case of C-type retroviruses, is dependent on cell division.

The MINT vector was initially constructed to minimize the possibility of NGFR functioning as a signal transducer in hematopoietic cells. However, no adverse effect was observed due to full-length NGFR expression on the growth of transduced TF1 cells (data not shown) or primary human hematopoietic cells assayed in vitro (see below). Because the titer of the MINT vector was somewhat lower than that of MINGFR or LINGFR vector (Fig 2A), we restricted our attention solely to the latter two vectors to compare directly the expression properties of MSCV- and MoMLV-based retroviral vector backbones after transduction of primary human hematopoietic precursors.

Transduction of human CD34$^+$Lin$^-$ cells. Enriched CD34$^+$ cells from BM or mPB were further purified by FACS to reach greater than 95% purity with respect to CD34 expression and lack of expression of lineage-specific differentiation markers (CD2 and CD4 for T cells, CD16 for natural killer [NK] cells, CD19 for B cells, CD14 for monocytes, CD15 for granulocytes, and glycophorin A for erythroid cells). These highly purified cell populations (denoted as CD34$^+$Lin$^-$) were prestimulated in a cytokine cocktail (IL-3, IL-6 plus SLF) overnight and then exposed to retroviral supernatants in the "spinoculation" protocol. Viral supernatants were added on days 1 and 2, and then the cells were cultured for 2 more days to allow gene expression. Gene transfer efficiency and NGFR expression level were then investigated by FACS analysis. Under the conditions used, total cell numbers increased during this period by approximately fourfold or twofold for input CD34$^+$Lin$^-$ cells isolated from BM (n = 3) or mPB (n = 6), respectively. Shown in Fig 3 as an example, ≈80% of the cells retained the CD34$^+$Lin$^-$ phenotype after 4 days in culture while the remaining cells lost the CD34 antigen and gained one or more lineage differentiation markers (n = 3 for mPB). The CD34$^+$ content of the cultures decreased rapidly thereafter, concomitant with the continued increase in total cell numbers. Among mPB cells which retained the CD34$^+$Lin$^-$ phenotype (gated in the R2 region in Fig 3), greater than 30% expressed the NGFR transgene at day 4, irrespective of which retroviral vector backbone was used. The percentages of NGFR$^+$ cells and the corresponding mean fluorescence intensities were, however, marginally but consistently higher for cells transduced by MINGFR than for those transduced by

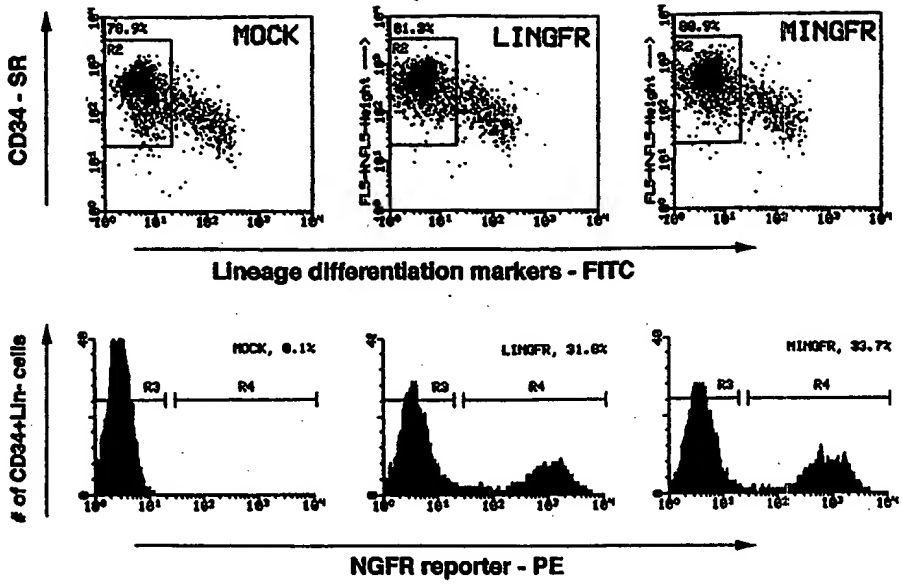


Fig 3. FACS analyses of NGFR transgene expression in transduced CD34⁺Lin⁻ cells. Sorted human mPB CD34⁺Lin⁻ cells were activated and transduced with LINGFR or MINGFR vector supernatants. Two days after viral transduction (4 days in culture), expression of CD34 (stained with an SR-conjugated antibody) and the Lin markers (stained with FITC-conjugated antibodies) were analyzed (dot plots in upper panels). Live cells which retained the CD34⁺Lin⁻ phenotype were gated (R2 regions) and percentages of gated cells among the total live cells were determined (≈80% in all cases). Efficiencies of gene transfer and expression levels in these CD34⁺Lin⁻ cell populations were assessed by presence of NGFR (stained by a PE-conjugated antibody) and are plotted as histograms in the lower panels. The gates set up to sort cells expressing NGFR (NGFR⁺, R4) and cells lacking the NGFR surface reporter (NGFR⁻, R3) are indicated as are the percentages of NGFR⁺ cells.

LINGFR (n = 3 for mPB). Similar results were obtained with transduced CD34⁺Lin-cells of BM origin (n = 2, data not shown).

Transduction of human CFC and CAFC progenitors. At day 2 posttransduction (4 days in culture), cells were plated in methylcellulose to detect clonogenic myeloid and erythroid progenitors in a standard CFC assay. Total numbers of colonies were first enumerated by visual inspection and then the efficiency of gene transduction was assessed either by PCR to detect the presence of the vector sequences in individually picked colonies or by FACS to examine NGFR expression in populations of CFC-derived myeloid and erythroid cells. Approximately 25% of the input cells transduced by either MINGFR or LINGFR formed colonies, similar to nontransduced controls (Table 1). PCR analysis indicated that 28% (out of 64 picked CFC colonies) were transduced by MINGFR and 34% (out of 65 picked CFC colonies) were transduced by LINGFR, indicating that both vectors were capable of achieving similar efficiencies of gene transfer to CFC. When NGFR expression in differentiated cells derived from CFC was analyzed after 2 weeks in culture, we found that only 7.7% to 12.5% of the total cell populations expressed detectable cell surface NGFR (Table 1).

We next sorted transduced CD34⁺Lin⁻ cells into two fractions on the basis of NGFR expression at day 2 posttransduction (Fig 3). The fact that a small percentage of NGFR⁻ cells were capable of generating NGFR⁺ differentiated cells in the CFC assays (by FACS, data not shown) illustrated that not all of the

Table 1. NGFR Transgene Expression in CFC-Derived Progeny

Cells	Vectors	CFC Frequency (%)	% of Individual Colonies That Show Transgene Presence (no.⁺/no. analyzed)	% of Pooled Cells That Show Transgene Expression at Day 14
Unsorted	MINGFR (81% CD34⁺Lin⁻, 34% NGFR⁺)	26.3 ± 0.9	28 (16/64)	12.5
	LINGFR (81% CD34⁺Lin⁻, 32% NGFR⁺)	25.1 ± 0.2	34 (22/65)	7.7
	None (81% CD34⁺Lin⁻)	23.6 ± 1.2	ND	0.5% (background)
Sorted (NGFR⁺CD34⁺Lin⁻)	MINGFR	22.9 ± 3.5	ND	78.0
	LINGFR	19.9 ± 0.9	ND	52.6

Two days after transduction using mPB CD34⁺Lin⁻ cells, unsorted and the transgene-expressing subset of CD34⁺Lin⁻ cells were each plated into methylcellulose culture. Colonies were enumerated at day 14. The presence of the retroviral DNA in individually picked colonies was determined by PCR, and transgene expression in CFC-derived cells was determined by flow cytometry of pooled colonies.

Abbreviation: ND, not done.

retrovially transduced cells expressed sufficient levels of NGFR transgene at day 2 posttransduction to permit their identification and isolation. Thus, the frequencies of NGFR$^+$ cells at this time point (day 2 posttransduction) were presumed to be underestimates of the actual values of cells containing the transgene. Nonetheless, since enrichment for cell-surface NGFR greatly simplified functional analyses of transduced cells and studies of LTR-mediated gene expression in functionally heterogeneous CD34$^+$Lin$^-$ cells, we subsequently focused on those NGFR$^+$ subpopulations that were clearly transduced and could be separated from untransduced cells. Approximately 20% of the CD34$^+$Lin$^-$NGFR$^+$ cells generated by transduction with MINGFR or LINGFR formed CFC colonies (Table 1), a rate slightly less than that of unsorted cells (which is ≈25%). Based on these plating efficiencies (20% v 25%), this result would indicate that the majority of transduced CFC progenitors by both vectors also expressed the NGFR transgene 2 days posttransduction. Interestingly, however, NGFR transgene expression in CFC-derived cells at the end of the 2 week CFC assay period are different among NGFR$^+$ cells transduced by the two vectors. Whereas 78% of CFC-derived progeny cells derived from MINGFR-transduced, sorted CD34$^+$Lin$^-$NGFR$^+$ cells continued to express the NGFR transgene after 2 weeks, approximately one half of cells derived from LINGFR-transduced NGFR$^+$ CFC had completely lost NGFR transgene expression while the remaining expressed NGFR at a reduced level (Table 1 and Fig 4). PCR analysis after cell sorting of LINGFR-transduced, CFC-derived cells which did not express NGFR after 2-week CFC assays confirmed that the transgene was still present in this NGFR$^-$ differentiated cell population (data not shown). Further multiparameter FACS analyses shown in Fig 4 indicated that downregulation of LINGFR-mediated NGFR expression occurred in differentiated erythroid (glycophorin A$^+$) cells and granulocytes (CD15$^+$), as well as in (CD14$^+$ cells) monocytes (data not shown). The downregulation of MoMLV LTR-mediated NGFR expression by the LINGFR vector in CD14$^+$ and CD15$^+$ myeloid cells was also seen in a suspension culture assay (data not shown). These findings are consistent with those of a recent report in which the MoMLV LTR-mediated transcription is downregulated in differentiated erythroid/myeloid progeny derived from transduced CD34$^+$ cells in CFC and suspension cultures, and the MSCV LTR-mediated transcription is substantially higher.[19]

Because silencing of MoMLV LTR-mediated expression had previously been observed in resting human T cells,[17] and in differentiated erythroid/myeloid cells as shown above, we next examined whether this phenomenon also occurred in differentiated CD19$^+$ B-lineage cells. The sorted NGFR$^+$ and NGFR$^-$ fractions of CD34$^+$Lin$^-$ cells (lacking CD19 expression) were plated at limiting dilution on stromal cell monolayers in a CAFC assay. There are two unique aspects of this long-term stromal-dependent CAFC assay compared with CFC assays: (1) CD19$^+$ B-lineage cells as well as myeloid cells are generated from CD34$^+$Lin$^-$ cells; and (2) late-appearing (after 5 weeks) cobblestone areas are considered to be derived from progenitors which are more primitive than CFC.[13,31] The frequencies of CAFC in the various transduced cell populations were calculated based on Poisson distribution (Fig 5A). The frequencies of CAFC present in the NGFR$^+$ subpopulations transduced by the MINGFR and LINGFR vectors were similar. However, both frequencies were more than 10-fold lower than the values calculated for the corresponding NGFR$^-$ subpopulations, indicating that the majority of CAFC were either not transduced at all or not expressing NGFR transgene 2 days posttransduction. An additional experiment using different preparations of viral supernatants confirmed this finding (data not shown).

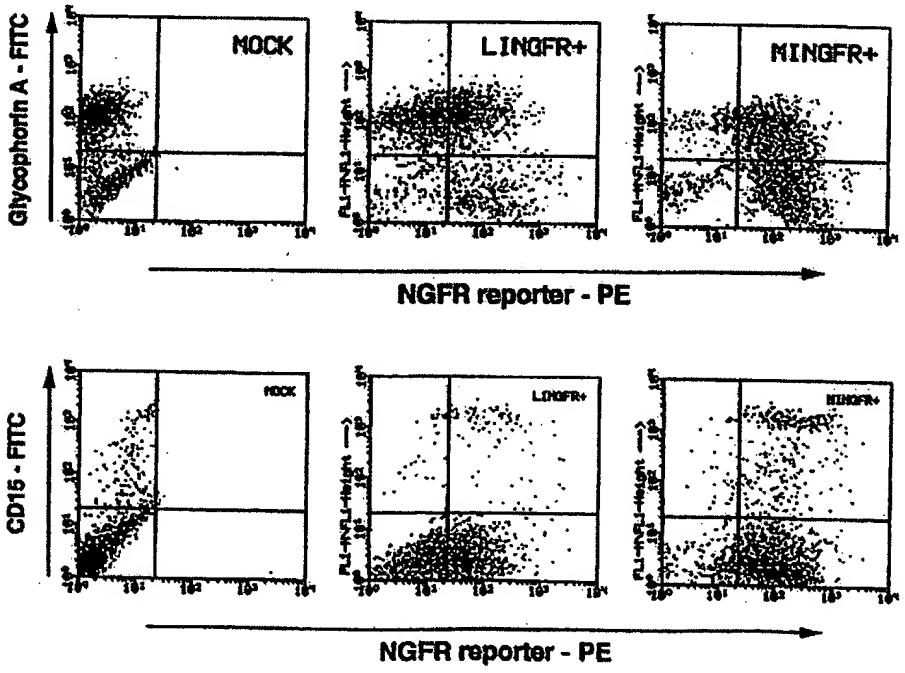
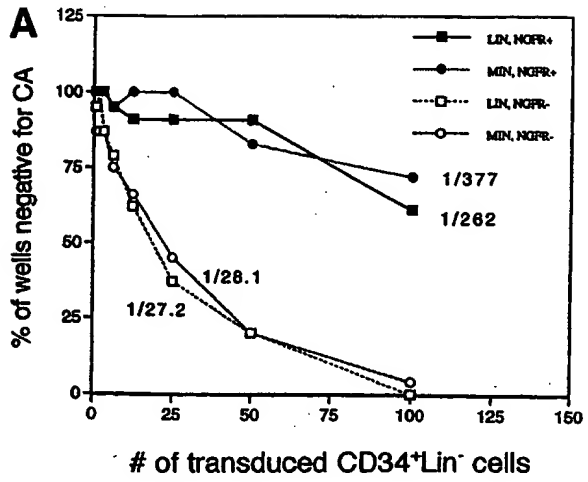


Fig 4. LTR-mediated NGFR transgene expression in progeny of transduced CFC. Sorted NGFR-expressing, CD34$^+$Lin$^-$ cells transduced with either the LINGFR (LINGFR$^+$) or MINGFR (MINGFR$^+$) vectors (see Fig 3) were assayed for CFC activity. After 14 days CFC numbers were enumerated (shown in Table 1), and total cells were obtained and stained for lineage markers and NGFR transgene expression. Fifty-two percent and 88% of erythroid cells (glycophorin A$^+$), and 71% and 91% of granulocytes (CD15$^+$) derived from LINGFR$^+$ and MINGFR$^+$ CFC, respectively, retained the NGFR reporter on cell surface.



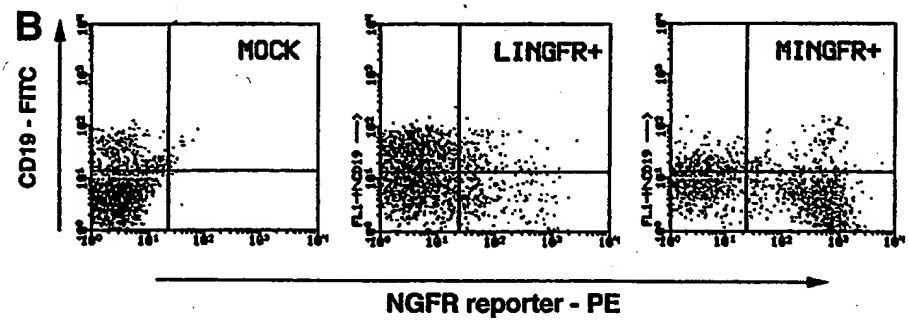


Fig 5. Maintenance of NGFR transgene expression in CAFC progeny. (A) Sorted NGFR-expressing, CD34⁺Lin⁻ cells transduced with either the LINGFR (LIN, NGFR⁺) or MINGFR (MIN, GFR⁺) vectors, or the sorted CD34⁺Lin⁻ cells lacking NGFR expression at day 2 posttransduction with LINGFR (LIN, NGFR⁻) or MINGFR (MIN, NGFR⁻) vectors (see Fig 3) were plated on SyS-1 stromal cell monolayers. Two-fold serial dilution of 100 CD34⁺Lin⁻ input cells were seeded per well and examined for cobblestone area (CA) formation weekly up to 6 weeks. The number of wells lacking any CA at week 5 were plotted as a function of numbers of sorted cells, respectively. The frequencies of CAFC were estimated based on Poisson distribution and the results are indicated. (B) NGFR transgene expression in B cells formed at week 6 of CAFC assays. CD19⁺ B cells (in addition to CD14⁺ and CD15⁺ myeloid cells) were generated from sorted CD34⁺Lin⁻NGFR⁺ cells in the presence of SyS-1 stromal cells as shown by the presence of the CD19 marker. Among CD19⁺ B cells, approximately 14% and 50% of the progeny cells derived from LINGFR-transduced (LINGFR⁺) and MINGFR-transduced (MINGFR⁺) CD34⁺Lin⁻NGFR⁺ cells, respectively, expressed the NGFR transgene at the end of CAFC assays.

NGFR transgene expression was examined in differentiated B cells at week 6 of CAFC assays. Cells in cobblestone area–containing wells initially seeded with preselected NGFR⁺ transduced cells were obtained and pooled for simultaneous FACS analysis of the CD19 B-cell marker and NGFR transgene expression (Fig 5B). The majority of harvested hematopoietic cells were myeloid cells. A population of CD19⁺ cells was detected in pooled CAFC⁺ wells containing transduced cells by either LINGFR or MINGFR vector, or mock-transduced cells (Fig 5B). Although ≈50% of CD19⁺ cells derived from MINGFR-transduced CD34⁺Lin⁻NGFR⁺ cells expressed a high level of NGFR reporter, a low to medium level of cell-surface NGFR could only be detected on ≈14% of CD19⁺ cells derived from LINGFR-transduced CD34⁺Lin⁻NGFR⁺ cells. Taken together, the results show that the MSCV LTR appears to be less susceptible to transcriptional silencing mechanisms than the MoMLV LTR in multiple lineages during in vitro differentiation of transduced human hematopoietic precursors.

Transduction of candidate human HSC assayed in SCID mice. It has been shown that subsets of CD34⁺Lin⁻ cells capable of long-term (>8 weeks) engraftment of fetal human bone implants in SCID mice (SCID-hu bone assay) exhibit B-lymphoid and myeloid potential as well as secondary repopulating capacity.[12,13] To assess whether transduced CD34⁺Lin⁻ cells expressing the NGFR reporter have candidate HSC activity, transduced mPB cells were tested in the SCID-hu bone assay for the presence of SCID repopulating cells (SRC). Because NGFR expression is higher in progeny derived from CD34⁺Lin⁻ cells transduced with MINGFR than with LINGFR in the in vitro assays described above, for SRC assays we restricted our attention to the MINGFR-transduced cells. Nine weeks postinjection, the presence of donor (MA2.1⁺) cells and the NGFR transgene expression was examined by FACS analysis of total cells obtained from the human bone implants (Fig 6). Cell-surface expression of NGFR could not be detected in nontransduced human hematopoietic cells (Fig 6A and B). The sorted CD34⁺Lin⁻NGFR⁺ cells that had been transduced by the MINGFR vector readily engrafted and maintained NGFR transgene expression in donor cells 9 weeks postinjection (Fig 6C and D).

The results of our first two experiments are summarized in Table 2. Nine of 10 injections with mock-transduced cells showed donor cell engraftment. Because this frequency (90%) is similar to historic data obtained using freshly isolated human hematopoietic precursors,[12] we believe that our protocol for ex vivo cell culture and transduction did not significantly alter the long-term SRC potential of CD34⁺Lin⁻ cells. Both NGFR⁺ and NGFR⁻ subpopulations selected at day 2 posttransduction by MINGFR engrafted (5 of 5 and 2 of 2, respectively). All 5 bone grafts that successfully repopulated with MINGFR-transduced, preselected CD34⁺Lin⁻NGFR⁺ cells showed reasonable levels of NGFR transgene expression (28% to 45% [37% ± 6%, n = 5] of the donor cells). In one experiment where sufficient numbers of donor-derived cells were available for an additional FACS analysis, both CD19⁺ (B-lineage) and

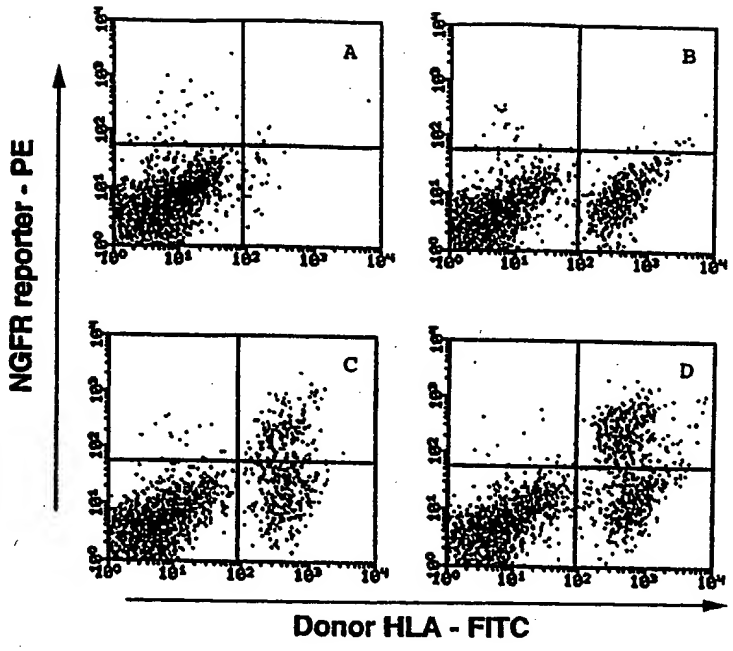


Fig 6. Representative FACS analyses of NGFR transgene expression in progeny of MINGFR-transduced, CD34⁺Lin⁻ cells recovered from SCID-hu bone mice. Harvested cells from implanted human bone fragments were stained with the FITC-conjugated MA2.1 antibody recognizing the donor cell's HLA, and the PE-conjugated anti-NGFR antibody recognizing the transgene expression on cell surface. Viable cells were then collected and analyzed by FACS. Bone fragments in the absence of injected cells (A) and the presence of mock-transduced cells (B) were included as controls to distinguish donor cells (MA2.1⁺) from endogenous human cells and/or contaminating murine cells (MA2.1⁻). Cells recovered from two different bone implants injected with sorted CD34⁺Lin⁻NGFR⁺ cells transduced with MINGFR are shown in (C) and (D). Approximately 35.5% (C) and 44.7% (D) of donor-derived cells were expressing the NGFR reporter after 9 weeks in vivo. See Materials and Methods for experimental details and Table 2 for a summary.

CD19⁻NGFR⁺ (transduced donor) cells were found (data not shown). These findings showed that the MSCV LTR is functional in primitive human hematopoietic precursors with SRC potential and remains active for long periods in their transduced progeny after in vivo differentiation.

DISCUSSION

In this report we evaluated the MSCV-based MINGFR vector carrying the NGFR reporter gene for its ability to efficiently transduce hematopoietic precursors purified from adult human BM or mPB. Based on FACS analyses and in vitro functional assays, similar efficiencies of gene transfer into CD34⁺Lin⁻ cells as well as CAFC and CFC progenitors were observed as for the MoMLV-based LINGFR vector. Moreover, stable gene transfer into HSC (or SRC), based on the NGFR reporter expression in engrafted donor cells, was observed in 7 of 7 grafts of MINGFR-transduced cells. Because of the qualitative nature of the SCID-hu mouse model (in the absence of a limiting dilution analysis which requires large numbers of engineered animals), it is difficult to estimate the frequencies of HSC/SRC-based gene transfer by MINGFR, and to what extent the improved retroviral vector stocks and the "spinoculation" transduction protocol used in this study contributed to the success of these experiments. Because others reported recently that inclusion of certain cytokines (eg, Flk2/Flt3 ligand) and the presence of cell extra-cellular matrix molecules (eg, fibronectin fragments) can further preserve/activate candidate HSC and increase efficiencies of gene transfer into them, we expect that the transduction protocol reported here can be further optimized with these molecules.[14,32,33]

Table 2. NGFR Transgene Expression in Transduced Human Cells Recovered From SCID-hu Bone Mice

Cells Tested	Engraftment Rate	% Donor Cells per Graft	% of Donor Cells That are NGFR⁺
Mock-transduced	9/10	8.5-45.7	N/A
NGFR⁺CD34⁺Lin⁻	5/5	11.6-43.2	28.4-44.7
NGFR⁻CD34⁺Lin⁻	2/2	14.4-29.4	7.6-12.6

Two days after transduction with the MINGFR retrovirus, CD34⁺Lin⁻ cells were sorted into NGFR-expressing (NGFR⁺) and NGFR⁻ subsets and injected into SCID-hu bone grafts. Nine weeks later grafts were analyzed by flow cytometry for donor-derived cells and NGFR transgene expression.

Abbreviation: N/A, not applicable.

Although the level of NGFR expression directed by the LTR of either vector was comparable in several cell lines and in the total CD34⁺Lin⁻ cell populations shortly after transduction, NGFR transgene expression mediated by MSCV LTR is substantially higher than that directed by MoMLV LTR in differentiated progeny derived from transduced CD34⁺Lin⁻ cells (Figs 4 and 5B). We observed that the LTR-mediated gene expression from LINGFR (which is based on the LXSN-type of MoMLV vector) was downregulated in differentiated cells belonging to multiple-lineages including erythroid, myeloid, and B-lymphoid cells. Similar observations with MoMLV vectors have been made by other investigators in erythroid/myeloid lineages,[19] in T cells,[17] and in mouse hematopoietic cells after in vivo BM repopulation.[18] Moreover, the phenomenon of the in vivo downregulation of gene expression of MoMLV LTR (typically caused by transcriptional silencing/inactivation) is not limited to the hematopoietic system, as it has also been observed in transduced primary fibroblasts, myoblasts, and hepatocytes in a variety of animal models.[34] However, it should be noted that others have documented MoMLV LTR-directed gene expression in the T-cell or myeloid progeny of transduced CD34⁺ cells purified from cord blood or fetal liver after repopulation of SCID-hu thymus or SCID-hu bone grafts.[35-37] Nonetheless, the possible extinction of MoMLV

LTR-mediated expression needs to be taken into account when sustained expression in multiple myeloid and lymphoid lineages is deemed necessary for therapeutic benefits in HSC-based gene transfer applications.

Based on the outcome of this study, it would appear that MSCV-based vectors may offer advantages over conventional MoMLV-based vectors for gene delivery to the human hematopoietic system. The ability to direct sustained high-level expression of exogenous genes in differentiated cells derived from transduced HSC/progenitors is obviously a desirable goal of a number of human gene therapy protocols targeting congenital blood disorders.[1] In addition, a vector that is permissive for expression in HSC may be of utility in those cancer gene therapy applications where the intent is to confer a drug-resistant phenotype to the patient's mature hematopoietic cells and their precusors to augment the therapeutic index of high-dose anti-tumor chemotherapy. In this regard, other types of retroviral vectors are currently being investigated for this purpose and are promising in human progenitor cells assayed in vitro.[38] It remains to be determined whether these vectors are also functional in directing gene expression in HSC/SRC and whether they are more active than MSCV.

The sensitivity of the NGFR reporter system allowed facile monitoring of transgene expression during differentiation of transduced human hematopoietic precursors into progeny cells belonging to multiple (myeloid and lymphoid) lineages.[22] Because the NGFR reporter gene is of human origin, it provides advantages in terms of increased specificity in SCID mouse models (compared with other mouse reporter genes in use) and, presumably, reduced immunogenicity in humans (compared with bacterial or mouse reporter genes). It was a concern at the outset that endogenous NGFR expression in human hematopoietic cells may complicate the SCID-hu bone assay. However, we have not been able to detect cell-surface NGFR expression in primary $CD34^+$ cells, their progeny cells differentiated in vitro and in vivo, or in fetal hematopoietic cells residing in the recipient bone fragments in any of our experiments. In any case, although the presence of the full-length NGFR gene did not exert any noticeable adverse effects on the transduced hematopoietic precursors we evaluated, use of vectors like MINT expressing C-terminally truncated NGFR genes should alleviate any remaining misgivings. Therefore, the stable and nonimmunogenic NGFR reporter coexpressed with a therapeutic gene from the same vector may be useful in human gene therapy based on human HSC as well as T cells,[39] if an easily detectable marker is desired to monitor and isolate transduced cells.

In summary, MSCV-based retroviral vectors encoding easily detectable and selectable markers should facilitate studies aimed at further characterizing the $CD34^+Lin^-$ subset containing SRC. It is anticipated that the information gained will lead to improvements in HSC-based gene and cellular therapies.

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EXHIBIT B

 Human ES Cells 18069302 Ama

Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells

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Human embryonic stem (ES) cells are pluripotent cell lines that have been derived from the inner cell mass (ICM) of blastocyst stage embryos [1–3]. They are characterized by their ability to be propagated indefinitely in culture as undifferentiated cells with a normal karyotype and can be induced to differentiate in vitro into various cell types [1, 2, 4–6]. Thus, human ES cells promise to serve as an unlimited cell source for transplantation. However, these unique cell lines tend to spontaneously differentiate in culture and therefore are difficult to maintain. Furthermore, colonies may contain several cell types and may be composed of cells other than pluripotent cells [1, 2, 6]. In order to overcome these difficulties and establish lines of cells with an undifferentiated phenotype, we have introduced a reporter gene that is regulated by a promoter of an ES cell-enriched gene into the cells. For the introduction of DNA into human ES cells, we have established a specific transfection protocol that is different from the one used for murine ES cells. Human ES cells were transfected with enhanced green fluorescence protein (EGFP), under the control of murine *Rex1* promoter. The transfected cells show high levels of GFP expression when in an undifferentiated state. As the cells differentiate, this expression is dramatically reduced in monolayer cultures as well as in the primitive endoderm of early stage (simple) embryoid bodies (EBs) and in mature EBs. The undifferentiated cells expressing GFP can be analyzed and sorted by using a Fluorescence Activated Cell Sorter (FACS). Thus, we have established lines of human ES cells in which only undifferentiated cells are fluorescent, and these cells can be followed and selected for in culture. We also propose that the pluripotent nature of the culture is made evident by the ability of the homogeneous cell population to form EBs. The ability to efficiently transfect human ES cells will provide the means to study and manipulate these cells for the purpose of basic and applied research.

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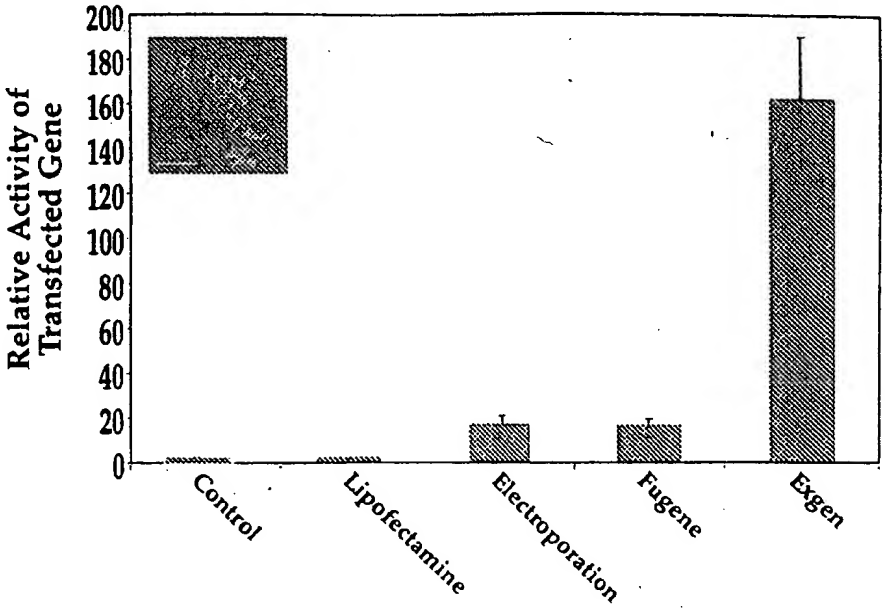
Results and discussion

The objective of this study was to obtain pure clones of human ES cells that are genetically modified so that their undifferentiated phenotype can be followed and selected for in vitro. Thus, we aimed at introducing the EGFP reporter gene under the control of a promoter of an ES cell-enriched gene into human ES cells. By tagging the undifferentiated cells with GFP, we wished to monitor the differentiation status of the cells in culture during growth and propagation as well as following spontaneous and induced differentiation. For this purpose, we chose to use the well-characterized promoter sequence of the murine *Rex1* gene [7]. *Rex1* is a retinoic acid-regulated zinc finger protein that is expressed in preimplantation mouse embryos (including the inner cell mass), trophoblast, and spermatocytes as well as in undifferentiated murine ES cells and some embryonic carcinoma (EC) cell lines [7, 8]. This gene is rapidly downregulated upon differentiation of the embryonic cells. Hence, by introducing *Rex1*-regulated gene markers (*Rex1*-EGFP) into human ES cells, we should be able to express these markers in pluripotent cells, allowing the determination of the differentiation status of these cells in culture.

In order to introduce *Rex1*-EGFP fusion gene into human ES cells, we had to establish a method to transfect the human embryonic cells with DNA. Although ES-like cell lines are now available from a large array of mammalian species (for a review, see Prelle et al. 1999 [9]), there are no published protocols for DNA transfection in any of the species, other than mice. In the mouse, electropora-

Figure 1



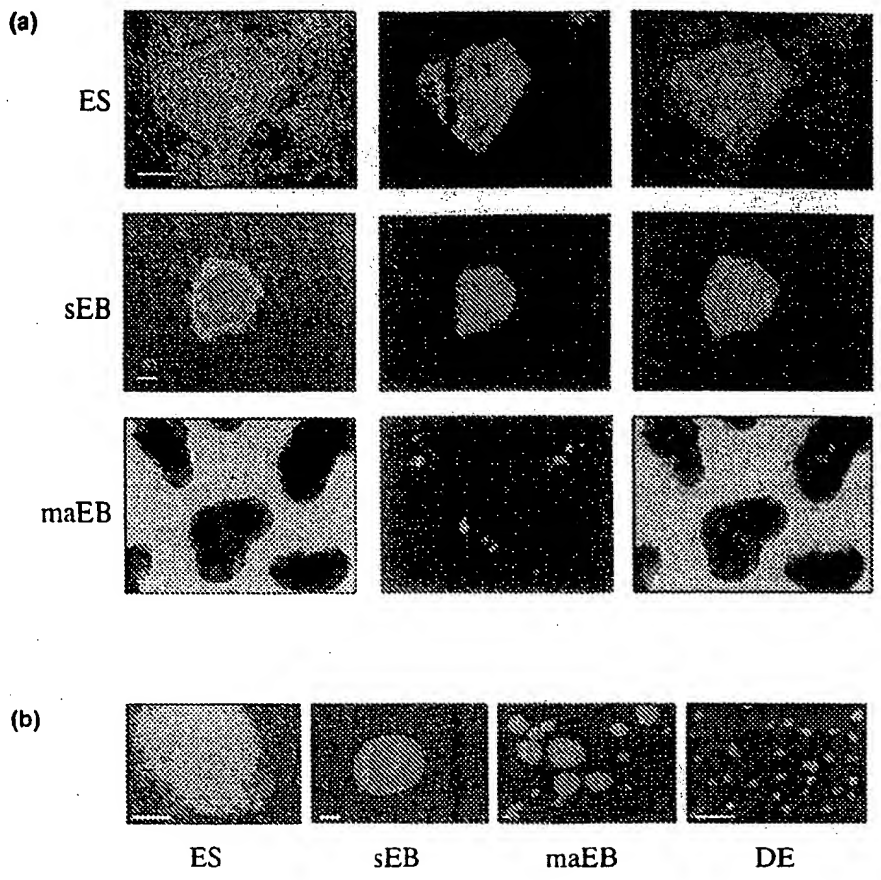
Transfection of DNA into human ES cells. DNA was introduced into human ES cells either by electroporation or by using several commercial reagents such as LipofectAMINE Plus (Life Technologies), FuGENE (Boehringer Mannheim), or ExGen 500 (Fermentas). To determine the efficiency of DNA introduction by each of the methods, the cells were transfected with a construct of firefly Rennila protein under the control of a TK promoter. The cells were harvested 48 hr after transfection, and luminosity of the Rennila protein was monitored using a luminometer. Results are given in the histogram as the relative activity of the transfected gene (luminosity units per mg of total protein), following the subtraction of the values obtained from samples of the appropriate MEF-only controls. Each experiment was repeated three times, and the mean with standard error is shown. Inset: human ES cells transiently transfected with EGFP under the control of the housekeeping gene E1F (elongation factor I) promoter. Note the green fluorescent ES cells that incorporated the foreign DNA. The scale bar indicates 100 μm.

tion was found to be the method of choice for introducing foreign DNA into ES cells [10]. However, human ES cells do not survive electroporation well. Therefore, we compared the efficiencies of several chemical-based methods for the transfection of H9 human ES cells [1] (passage 40–50). Initially, an expression construct of EGFP under the control of the housekeeping gene elongation factor I (E1F) was introduced into human ES cells by several different reagents. Transient expression of the GFP was observed in no more than 10% of the cells, mainly by the human ES cells, and not by the feeder layer of mouse embryonic fibroblasts (MEF) (over 80% of the fluorescent cells had ES cell morphology and resided within the colony boundaries) (Figure 1, inset). To allow quantification and comparison of transfection efficiencies between protocols, a TK-firefly Renilla luciferase reporter gene (Dual Luc Reporter Assay Kit, Promega) was introduced into growing colonies of human ES cells, either by LipofectAMINE Plus (Life Technologies), FuGENE (Boehringer Mannheim), or ExGen 500 (Fermentas) (performed according to the manufacturer's protocols). Cell samples were lysed (using the passive lysis buffer of the assay kit) and evaluated for the efficiency of transient transfection by measuring the relative activity of luciferase in respect to protein concentration (as determined by the Bradford method [BIO-RAD Protein Assay]) 48 hours after transfection. A clear difference between ExGen 500, FuGENE, LipofectAMINE Plus, and electroporation was apparent. Transfection with ExGen 500 seems to deliver DNA into human ES cells in an order of magnitude more efficiently than other reagents that we have examined (Figure 1).

Using the transfection protocol of ExGen 500, a *Rex1*-EGFP expression vector, which includes the neo selectable marker, was delivered into human ES cells. The following day, cells were trypsinized and replated on a feeder of inactivated MEF that was resistant to neomycin (MEF^{Neo+}), allowing the clonal propagation of transfected cells by G418 selection. At 14 days in culture, neomycin-resistant fluorescent colonies were isolated and propagated for several passages while maintaining their level of fluorescence (up to 13 passages), allowing the establishment of individual cell lines. In our experience, stable clones were derived in an efficiency of $\sim 10^{-5}$ of the transfected cells.

Of the various neo resistant colonies, we have established 10 cell lines, 4 of which were examined under different culture conditions (Figure 2). When grown on feeder cells in the presence of leukemia inhibitory factor (LIF) (to support undifferentiated growth), high expression of GFP was detected in the small and densely packed cells of the undifferentiated colony. The fluorescent emission overlaps well with the discrete margins of the colony and is absent in the periphery, where spontaneous differentiation takes place (Figure 2a). When the transfected human ES cells were induced to differentiate by growing as cell aggregates in suspension culture, fluorescence gradually declines, initially, in the outer surface of 4 day old simple

Figure 2

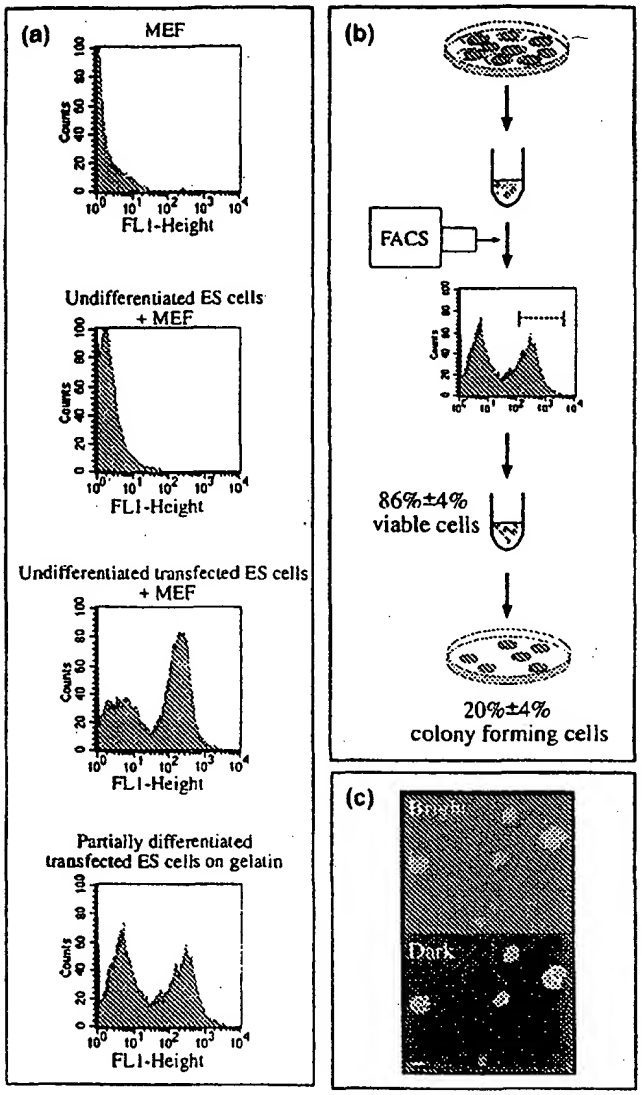


Isolation of human ES clones transfected with a marker for undifferentiated cells. **(a)** Human ES cells underwent stable transfection with EGFP fused to the murine *Rex1* minimal promoter sequence. The transfected ES cells and their differentiated cell derivatives are shown: simple embryoid body (sEB), and mature embryoid bodies (maEBs). The left and middle columns are photos of bright and dark fields, respectively. The right column is the overlay of the two photos. Note that only the undifferentiated cells are fluorescent. The fluorescent ES colony is surrounded by differentiated nonfluorescent cells. The simple EB is labeled only in the middle and not in the peripheral primitive endodermal cells [11]. Mature EBs are generally not fluorescent, and only very distinct areas in them are still fluorescent (probably residual undifferentiated cells). The scale bar indicates 100 μm. **(b)** The stable transfection of human ES cells with a constitutively expressed EGFP construct, driven by the mouse *PGK* promoter. Overlay photos of the dark on bright field of the transfected ES cells (ES) and their differentiated cell derivatives are shown: simple embryoid bodies (sEB), mature embryoid bodies (maEB), and differentiating embryonic cells derived from dissociated embryoid bodies (DE). Note that GFP is expressed by all cells, differentiated and undifferentiated, in the proliferating ES colony as well as by all cells of simple and mature EBs (including those in the outer layer of the sEB, where differentiation of primitive endoderm is taking place in the mouse EBs [11]). The scale bar indicates 100 μm.

EBs, where a layer of primitive endoderm was demonstrated in the mouse [11]. Later, if maintained to form mature EBs (20 days in suspension), the fluorescence practically ceases, apart from a few cores of undifferentiated cells (Figure 2a). This is in contrast to transfections of constructs driven by constitutively expressed promoters (*PGK*, phosphoglycerate kinase 1; and *CMV*, cytomegalovirus), in which expression of GFP was observed in both undifferentiated and differentiated cells of the colony (Figure 2b).

In an attempt to distinguish between populations of undifferentiated and differentiated human ES cells, we have analyzed the *Rex1*-EGFP transfected cell lines by FACS (Figure 3). Cell samples of MEF, undifferentiated human ES cells, and a mixture of undifferentiated and differentiated transfected cell lines were characterized according to their fluorescent emission. As expected, a clear difference in fluorescent intensity exists between the undifferentiated cultures of untransfected and transfected cell lines. In addition, when comparing EGFP-transfected human ES cells to their differentiated derivatives, a reduction in emission intensity is observed (Figure 3a). This shift in fluorescence emission represents a transition, from undifferentiated to differentiated, in the state of the cells. To allow the collection and selective propagation of the most fluorescent cells in the culture, three different GFP-expressing human ES cell lines (3–4 cell sample replicates per clone) were sorted by FACS (Figure 3b). Cell sorting was performed according to the background level of fluorescence that had been obtained by the analysis of untransfected human ES cells. The different cell samples were individually sorted for collection into tubes containing 25,000–50,000 cells each. By comparing the total cell count and the number of viable cells prior and following cell sorting (determined by trypan blue staining), we could show that the FACS procedure had no detrimental effect on cell viability, as 86% of the sorted cells were viable. Moreover, by plating the isolated cells on MEF^{Neo+} 10 cm^2 culture dishes and allowing their propagation in vitro, we demonstrated their ability to develop into undifferentiated fluorescent-labeled human ES colonies, with an efficiency of 20% ± 4% (n = 11) (ranging from 2% to 41%) (Figure 3b). In our procedure, many sorted cells were grown in the same culture dish, potentially allowing

Figure 3



FACS analysis and cell sorting of the transfected human ES cells. **(a)** Human ES cells transfected with *Rex1*-EGFP construct were analyzed by FACS according to the intensity of green fluorescence emission (FL1 height). Cell samples of MEF and undifferentiated human ES cells were used as controls. Fluorescent intensity between undifferentiated human ES cells, transfected human ES cells, and their differentiated cell culture derivatives (obtained by growth on gelatin-coated plates in the absence of LIF and bFGF) was then compared. The high-fluorescent intensity peak represents GFP positive cells, while the low-intensity peak represents background levels that may result either from autofluorescence or residual promoter activity. **(b)** Cell sorting of three GFP-expressing cell lines was performed by FACS. Following trypsin digestion, cell samples (3–4 replicates per clone) were evaluated for percentage of cell viability (84%) and sorted according to the intensity of green fluorescent emission. The collected cell samples (25,000–50,000) were redetermined for cell viability (86%) and replated on MEF^{Neo+} culture dishes (2,500–8,000 cells per dish). Following growth in vitro, cell culture dishes were inspected and recorded for total number of proliferating human ES colonies (20% ± 4% [n = 11]). **(c)** Photos of fluorescent-labeled proliferating human ES colonies (top, bright field; and bottom, dark field) obtained 4 days after cell sorting by FACS.

mutual support of growth and relatively high plating efficiency. This differs from the single cell dilution procedure by which the clonality of human ES cells was conferred [6]. After FACS sorting, the cells have a morphology indistinguishable from that seen before, but we have not yet tested them for pluripotency.

In our research, we have developed a stem cell selection approach in an attempt to facilitate maintenance of human ES cells in vitro. Currently, the available methods applied for this purpose involve the identification and isolation of single colonies under a dissecting microscope; however, these procedures are time consuming and labor intensive. As an alternative, we suggest a method for purifying undifferentiated cells by cell sorting the fluorescent-labeled cells from a mixed population. Similar selection of undifferentiated clones may be achieved by introducing into the cells a gene that enables drug selection, such as neo resistance gene, under the regulation of an ES-specific promoter [12]. By generating pure populations of undifferentiated cells, as described above, we should be able to avoid the loss of human ES cultures due to their spontaneous differentiation in vitro. Our system of introducing a cell-specific selectable marker into the genome of undifferentiated human ES cells provides a model for isolating specific cell types for transplantation from heterogeneous cell cultures obtained by induced differentiation. Similarly, such methods may be considered for eliminating human ES cells by negative selection prior to transplantation of differentiated cells, avoiding the risk of tumor induction.

The expression of *Rex1*-regulated reporter gene by the cells in the growing colony illustrates that these cells maintained their undifferentiated phenotype. In addition, the transfected cells can develop into undifferentiated colonies that maintain their ability to form EBs in vitro. These results support previous work that demonstrated the clonality of human ES cells [6] and the capacity of these homogenous cultures to differentiate into the three germ layers.

Finally, we report the first isolation of genetically engineered human ES cell lines and describe an efficient protocol for transfecting these cells. By introducing genetic modifications into their genome, we should be able to manipulate them in vitro and use them as vectors in cell-based therapies as well as for other biomedical and research purposes.

Materials and methods

Cell culture

Human ES cells (H9 [1], passage 40–50) were cultured on a Mitomycin-C-treated mouse embryonic fibroblast (MEF) feeder layer (obtained from 13.5 day embryos) in 80% KnockOut DMEM medium (GIBCO-BRL), supplemented with 20% KnockOut SR (a serum-free formulation) (GIBCO-BRL), 1 mM glutamine (GIBCO-BRL), 0.1 mM β-mercaptoethanol (Sigma), 1% nonessential amino acids stock (GIBCO-BRL), Penicillin (50 units/ml), Streptomycin (50 μg/ml), and 4 ng/ml basic fibroblast

growth factor (bFGF). The cells were grown in the presence of LIF (10^3 units/ml, GIBCO-BRL), although its necessity for supporting undifferentiated growth in human ES cells is currently unclear [1, 2]. The undifferentiated cell cultures were induced to differentiate in vitro into EBs by omitting LIF and bFGF from the growth media and allowing aggregation in petri dishes [3]. Following the formation of simple EBs by a 5 day cell aggregation step, cell masses were either trypsin dissociated and left to grow as a monolayer on fibronectin-creating cultures of differentiated embryonic (DE) cells [5] or further expanded in suspension and allowed to develop into 20 day old mature EBs (maEBs) (yielding cavitated and cystic EBs). In addition, we allowed some undifferentiated cells to undergo spontaneous differentiation as a monolayer by growing them on 0.1% gelatin-coated plates (Merck) in the absence of LIF and bFGF.

Plasmid construction

Rex1-EGFP and *PGK*-EGFP expression vectors were constructed by the deletion of the *CMV* promoter sequence from pEGFP-N1 (Clontech) and the insertion of either the mouse *Rex1* promoter sequence (700 bp) into the HindIII restriction site or the mouse *PGK* (phosphoglycerate kinase 1) promoter (515 bp) into the EcoRI and BamHI restriction sites. These constructs contained an SV40-driven neo selectable marker. The use of SV40 promoter in our system was sufficient to confer G418 resistance by driving the neo gene, although it was somewhat inefficient in mouse ES cells.

Transfection and establishment of transgenic cell lines

Fully expanded and undifferentiated human ES cells underwent stable transfection with *Rex1*-EGFP, *CMV*-EGFP, or *PGK*-EGFP plasmid DNA by the ExGen 500 transfection system (Fermentas). Transfection of human ES cells was carried out in 6-well trays on MEF, two days after plating, and was performed as described by the manufacturer's protocol. Specifically, 2 μg of plasmid DNA plus 10 μl of the transfecting agent ExGen 500 were added to ~3×10^5 cells in a final volume of 1 ml media per well. The cells were centrifuged at 280 × g for 5 min and incubated at 37°C in a moist chamber for an additional 45 min. Residuals of the transfecting agent were removed by washing the cells twice with PBS. The following day, the cells were trypsinized and ~5×10^5 were replated on each 10 cm culture dish containing inactivated MEF^{Neo+}. Two days following replating, G418 (200 ng/ml) was administered to the growth medium, allowing the selective propagation of transfected cells in culture. By day 14, neo resistant fluorescent-labeled colonies were identified by a fluorescent microscope (up to 10 colonies per plate). Using our constructs, over 80% of the neo resistant colonies were also GFP positive. Single transgenic colonies were picked by a micropipette, dissociated into small clumps of cells, and transferred into a 24-well culture dish on a fresh feeder of MEF^{Neo+}. The cells continuously proliferated in the presence of G418 and formed a large number of expanding undifferentiated colonies.

FACS analysis and cell sorting

FACS analysis of *Rex1*-EGFP-expressing cells was performed on a FACSCalibur system (Becton-Dickenson), according to their green fluorescent emission. Undifferentiated human ES cells were used to set the background level of fluorescence. Transfected cells, either undifferentiated (grown on MEF cells in the presence of LIF) or partially differentiated (obtained by growth on gelatin in the absence of LIF and bFGF) were analyzed for fluorescence intensity and compared to control cells.

GFP-expressing cell lines were sorted by FACS according to their fluorescence emission. Following trypsin digestion and centrifugation, cell pellets (~$2-5\times10^6$ cells from each cell line) were resuspended in PBS, filtered by a 70 μM cell strainer (Falcon), and divided into four different tubes, which were kept on ice under sterile conditions. Total cell counts and percent of viable cells were determined for each sample by 0.5% trypan blue staining (1:1 volume) prior to analysis and sorting by FACS. Cell samples were sorted for the collection of 25,000–50,000 cells in 50 ml conical tubes (Falcon) precoated with BSA (4% in PBS). Following centrifugation (5 min, 1000 rpm), the cells were resuspended in 0.5 ml PBS, analyzed for cell viability as described above, and plated on MEF^{Neo-} 10 cm culture dishes. Following 4 days in culture in the presence of G418 (200 ng/ml), the cell cultures were inspected under the microscope, and the total number of colonies per plate was recorded.

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EXHIBIT C

Homologous recombination in human embryonic stem cells

Thomas P. Zwaka and James A. Thomson*

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Homologous recombination applied to mouse embryonic stem (ES) cells has revolutionized the study of gene function in mammals[1–4]. Although most often used to generate knockout mice, homologous recombination has also been applied in mouse ES cells allowed to differentiate *in vitro*. Homologous recombination is an essential technique if human ES cells[5] are to fulfill their promise as a basic research tool. It also has important implications for ES cell–based transplantation and gene therapies. Significant differences between mouse and human ES cells have hampered the development of homologous recombination in human ES cells. High, stable transfection efficiencies in human ES cells have been difficult to achieve, and, in particular, electroporation protocols established for mouse ES cells work poorly in human ES cells[6]. Also, in contrast to their murine counterparts, human ES cells cannot be cloned efficiently from single cells, making it difficult to screen for rare recombination events[7]. Here we report an electroporation approach, based on the physical characteristics of human ES cells, that we used to successfully target *HPRT1*, the gene encoding hypoxanthine phosphoribosyltransferase-1 (HPRT1), and *POU5F1*, the gene encoding octamer-binding transcription factor 4 (Oct4; also known as POU domain, class 5, transcription factor 1 (POU5F1)).

The *HPRT1* gene is located on the X chromosome, so a single homologous recombination event leads to complete loss of function in XY cells. HPRT1-deficient cells can be selected based on their resistance to 2-amino-6-captopurine (6-TG), and thus the frequency of homologous recombination events is easy to estimate[8]. Because of these properties, HRPT1 played an important role in the initial development of homologous recombination in mouse ES cells[4,9]. We designed an *HPRT1*-targeting vector that contains a short homologous arm (1.9 kb) on the 5′ side of exon 7 and a long homologous arm (10 kb) on the 3′ side of exon 9, which deletes regions of the last three exons (Fig. 1A). A neomycin resistance (*neo*) cassette was inserted between the two homologous arms, and at the end of the 3′ homologous arm, the thymidine kinase gene (*tk*) was added to allow negative selection with gancyclovir.

For human ES cells, the best chemical reagents yield stable (drug-selectable) transfectants at rates of about 10^{-5}; mouse ES cell electroporation procedures yield substantially lower rates[6]. Given the very low transfection rates previously reported for electroporation, we first tested two chemical transfection reagents (ExGen 500 versus FuGene-6) for homologous recombination of the *HPRT1* locus in human ES cells. Although clones were obtained using both transfection reagents—for ExGen and FuGene respectively, 130 versus 261 G418-resistant clones and 35 versus 61 gancyclovir-resistant clones—none of these were resistant to both G418 and 6-TG (*HPRT1*$^-$), indicating that none were the result of homologous recombination. These results are consistent with the observation that transfection using lipid (FuGene-6; Roche, Indianapolis, IN) and cationic (ExGen 500; Fermentas, Hanover, MD) reagents results in inefficient homologous recombination in other mammalian cell types, and that physical methods of introducing DNA are, in general, more effective[10].

Our failure to achieve homologous recombination with chemical transfection reagents led us to re-evaluate electroporation procedures for human ES cells. In our hands, electroporation using a typical mouse ES cell protocol[11] (220 V, 960 μF, electroporation in PBS) yielded a stable transfection rate of ~10^{-7}. Given current culture techniques, this frequency is too low to be practical for identifying rare homologous recombination events. As human ES cells are significantly larger than mouse ES cells (~14 μm versus ~8 μm), we tried electroporation parameters described for larger cells. Also, because our current culture conditions allow only about 1% of individual human ES cells to survive and form colonies when plated at low densities, we electroporated the ES cells in clumps, not as individual cells, and plated them out at high densities. Additionally, we electroporated the cells in an isotonic, protein-rich solution (standard cell culture medium), instead of PBS, at room temperature. Using this modified protocol, we were able to obtain stable, G418-resistant clones at transfection rates that were 100-fold (or more) higher than those attained with standard mouse ES cell electroporation procedures. After transfection of 1.5×10^7 cells with the linearized *HPRT1*-targeting vector, we obtained 350 G418-resistant clones. Of these, 50 were resistant to gancyclovir, and of these, 7 were also resistant to 6-TG, suggesting successful homologous recombination. Polymerase chain reaction (PCR) and Southern blotting (Fig. 1B) confirmed that homologous recombination had occurred in all of these 6-TG-resistant clones.

One of the uses of homologous recombination in human ES cells will be to generate 'knock-in' cell lines with a selectable marker introduced into a locus with a tissue-specific expression pattern. Such knock-ins will be useful, for example, to purify a specific ES cell–derived cell type from a mixed population[12,13]. To test this approach, we introduced two reporter genes into the Oct4-encoding gene *POU5F1* by homologous recombination. Oct4, which belongs to the POU (Pit, Oct, Unc) family of transcription factors[14], is expressed exclusively in the pluripotent cells of the embryo and is a central regulator of pluripotency[14,15]. We introduced two promoterless reporter-selection cassettes into the 3′ untranslated region (UTR) of *POU5F1*. The first cassette contained an internal ribosomal entry site (IRES) sequence of the encephalomyocarditis virus and the gene *EGFP*, encoding the enhanced green fluorescence protein (EGFP). The second cassette included the same IRES sequence and the gene *neo*, encoding neomycin resistance. The cassettes were flanked by two homologous arms (Fig. 2A). After electroporation of 1.5×10^7 human ES cells with the linearized targeting vector (Fig. 2A), we obtained 103 G418-resistant clones. PCR (Fig. 2B, left) and DNA Southern blotting (Fig. 2B, right) demonstrated that 28 of these clones (27%) were positive for homologous recombination. Using a second targeting vector with a longer 3′ homologous arm, we obtained a higher rate of homologous recombination, almost 40% (22 homologous clones out of 56 G418-resistant clones). Similar transfection experiments using FuGene-6 with the same *POU5F1*-targeting vector resulted in 11 G418-resistant clones, none of which resulted from homologous recombination.

Human ES cells with the *POU5F1* knock-in expressed EGFP (Fig. 2C), which turned off during differentiation. Both drug selection and flow cytometry to detect EGFP expression (Fig. 2D) allowed purification of undifferentiated ES cells from a mixed, partially differentiated cell population. These properties will make the knock-in cell lines useful for studying *POU5F1* gene expression during differentiation *in vitro* and for optimizing culture conditions for human ES cells.

Electroporation of human ES cells with a DNA construct containing a *neo* cassette under the control of the *tk* promoter yielded a stable

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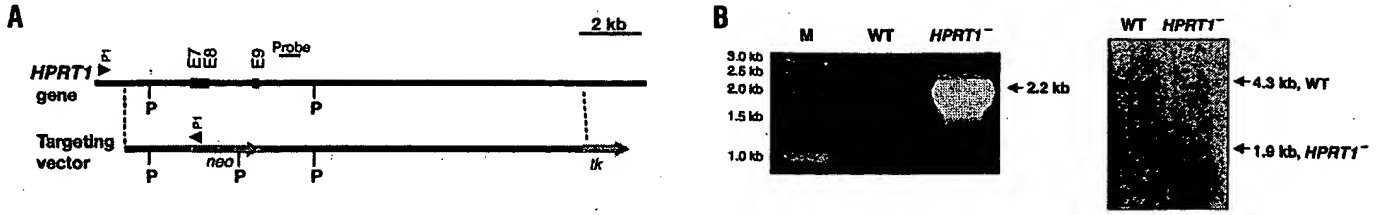

Figure 1. Targeted deletion of the last three exons of the *HPRT1* gene. (A) Partial structure of human *HPRT1* and the gene-targeting vector. 3′ probe for Southern blot analysis is shown. E, exon; P, *Pst*I; P1, primer pair; *tk*, thymidine kinase gene. (B) PCR analysis of ES cell lines with P1 (left) and Southern blot analysis with dedicated probe and *Pst*I digest (right). *HPRT1*⁻, knockout; WT, wild-type cells; M, marker.

transfection rate of 5.6×10^{-5}, giving an estimated 26:1 ratio of stable transfected clones to homologous recombination events for the first *POU5F1* construct. Similarly, for transfection of the *HPRT1* vector, the ratio of G418-resistant clones to HPRT1⁻ clones was 50:1. These targeting ratios for both HPRT1 and Oct4 are comparable to those observed for mouse ES cells[16], and suggest that although successful transfection strategies differ between human and mouse ES cells, the frequency of homologous recombination itself may be similar. However, it will be important to determine whether this similarity of rates between human and mouse ES cells holds true for genes not expressed in ES cells.

Homologous recombination in human ES cells will be important both for elucidating gene function *in vitro* and for modifying specific ES cell–derived tissues for therapeutic applications in transplantation medicine. For therapeutic applications, controlled modification of specific genes should be useful for purifying specific ES cell–derived differentiated cell types from a mixed population, for altering the antigenicity of cells, and for giving cells new properties (such as viral resistance) to combat specific diseases. Homologous recombination in human ES cells might also be used for recently described approaches combining therapeutic cloning with gene therapy[17]. Modifying specific genes for *in vitro* studies will be important for learning more about the pathogenesis of diseases for which mouse models have proven inadequate. For example, Hprt1-deficient mice do not show a phenotype similar to Lesch-Nyhan syndrome, the condition that results from HPRT1 deficiency in humans[18]. *In vitro* neural differentiation of HPRT1⁻ human ES cells or transplantation of ES cell–derived neural tissue to an animal model[19] could help clarify the pathogenesis of Lesch-Nyhan syndrome. Indeed, homologous recombination and human ES cells offer a promising approach for understanding the function of any human gene, and this approach will be particularly important for human genes that differ in clinically significant ways from the corresponding mouse genes.

Experimental protocol

HPRT1 knockout. The gene-targeting vector was constructed by replacement of the last three exons (exon 7, 8, and 9) of the *HPRT1* gene with a *neo* cassette under the control of the *tk* promoter. This cassette is flanked in the 5′ direction by a 10 kb homologous arm and in the 3′ direction by a 1.9 kb homologous arm. Isogenic homologous DNA was obtained by long-distance genomic PCR and subcloned. H1.1 human ES cells were cultured as described[5]. One week before electroporation, cells were plated onto Matrigel (Becton Dickinson, San Jose, CA) and cultured with fibroblast-conditioned medium[20]. To remove colonies as intact clumps, human ES cell cultures were treated with collagenase

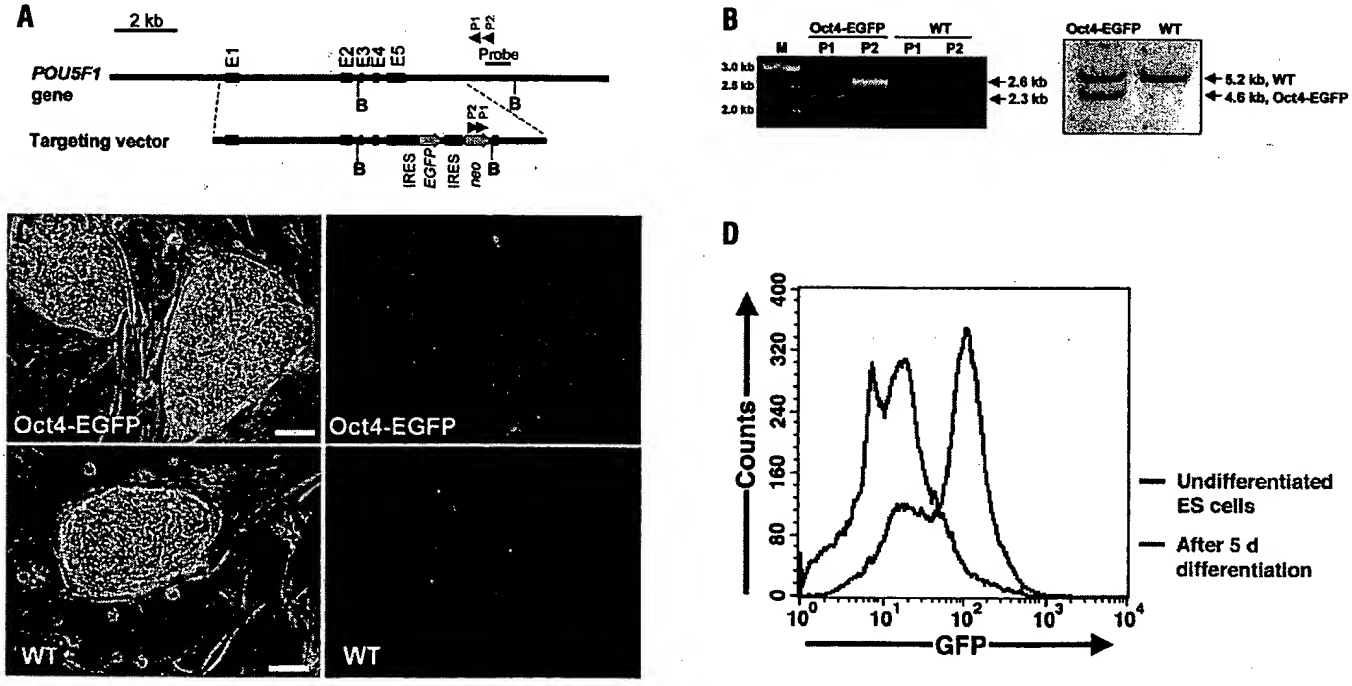

Figure 2. Targeting of an IRES-*EGFP*-IRES-*neo* cassette into the 3′ UTR of the gene *POU5F1*, which encodes Oct4. (A) Partial structure of the human *POU5F1* gene and the gene-targeting vector. 3′ probe for Southern blot analysis is shown. E, exon; B, *Bam*HI; P1 and P2, primer pairs 1 and 2. (B) PCR analysis of ES cell lines with P1 and P2 (left) and Southern blot analysis with dedicated probe and *Bam*HI digest (right). *POU5F1-EGFP*, heterozygous knock-in; WT, wild-type cells; M, marker. (C) Fluorescence microscopy (right) and phase-contrast microscopy (left) of *POU5F1* knock-in and wild-type colonies. Bar, 25 μm. (D) Flow cytometry of *POU5F1* knock-in undifferentiated (EGFP-positive) ES cells (blue) and their differentiated derivates after 5 d of differentiation (red).

IV (1 mg/ml; Invitrogen, Carlsbad, CA) for 7 min, washed with medium, and resuspended in 0.5 ml culture medium ($1.5–3.0 \times 10^7$ cells). Just before electroporation, 0.3 ml PBS (Invitrogen) containing 40 μg linearized targeting vector DNA was added. Cells were then exposed to a single 320 V, 200 μF pulse at room temperature using the BioRad Gene Pulser II (0.4 cm gap cuvette; BioRad, Hercules, CA). Cells were incubated for 10 min at room temperature and were plated at high density on one 10 cm culture dish coated with Matrigel. G418 selection (50 μg/ml, Invitrogen) was started 48 h after electroporation. After one week, G418 concentration was doubled and 6-TG selection (1 mM; Sigma, St. Louis, MO) was started. After three weeks, surviving colonies were analyzed individually by PCR using primers specific for the *neo* cassette and for the *HRPT1* gene just upstream of the 5′ homologous region, respectively. PCR-positive clones were rescreened by Southern blot analysis using *Pst*I-digested DNA and a probe on the 3′ side of the *neo* cassette.

***POU5F1* knock-in.** The gene-targeting vector was constructed by insertion of an IRES-*EGFP*, an IRES-*neo*, and an SV40 polyadenylation sequence (approximately 3.2 kb) into the 3′ untranslated region of the fifth exon of the human *POU5F1* gene. This cassette is flanked in the 5′ direction by a 6.3 kb homologous arm and in the 3′ direction by a 1.6 kb (6.5 kb in an alternative targeting vector) homologous arm. Isogenic homologous DNA was obtained by long-distance genomic PCR and subcloned. H1.1 human ES cells were cultured as described[5]. When an alternative targeting vector with a longer (6.5 kb) 3′ homologous arm was used, the rate of homologous recombination increased to almost 40% (22 homologous clones out of 56 stable clones).

Flow cytometry. Before flow cytometry, ES cell differentiation was induced by incubating the cells for 5 d in unconditioned medium on Matrigel. ES cells were treated with trypsin-EDTA and washed with PBS (both from Invitrogen). Dead cells were excluded from analysis by forward- and side-scatter gating. Samples were analyzed using a FACScan (Becton Dickinson) flow cytometer and Cellquest software (Becton Dickinson). A minimum of 50,000 events was acquired for each sample.

Acknowledgments

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Competing interests statement

The authors declare that they have no competing financial interests.

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Site-specific cassette exchange and germline transmission with mouse ES cells expressing ϕC31 integrase

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Currently two site-specific recombinases are available for engineering the mouse genome: Cre from P1 phage[1,2] and Flp from yeast[3,4]. Both enzymes catalyze recombination between two 34–base pair recognition sites, *lox* and *FRT*, respectively, resulting in excision, inversion, or translocation of DNA sequences depending upon the location and the orientation of the recognition sites[5,6]. Furthermore, strategies have been designed to achieve site-specific insertion or cassette exchange[7–10]. The problem with both recombinase systems is that when they insert a circular DNA into the genome (*trans* event), two *cis*-positioned recognition sites are created, which are immediate substrates for excision. To stabilize the *trans* event, functional mutant recognition sites had to be identified[8–12]. None of the systems, however, allowed efficient selection-free identification of insertion or cassette exchange. Recently, an integrase from *Streptomyces* phage ϕC31 has been shown to function in *Schizosaccharomyces pombe*[13] and mammalian[14,15] cells. This enzyme recombines between two heterotypic sites: *attB* and *attP*. The product sites of the recombination event (*attL* and *attR*) are not substrates for the integrase[16]. Therefore, the ϕC31 integrase is ideal to facilitate site-specific insertions into the mammalian genome.

Here we demonstrate that the ϕC31 integrase system is compatible with embryonic stem (ES) cell–mediated genomic alterations in the mouse and is particularly useful to achieve site-specific transgene insertions or

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EXHIBIT D

Neural Grafts

AmB

British Journal of Neurosurgery (1995) 9, 303–317

REVIEW ARTICLE

Neural injury repair: hope for the future as barriers to effective CNS regeneration become clearer

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Abstract

In this review the author outlines the early history of clinical and scientific research upon the inability of the CNS in man to successfully regenerate following injury. As we proceed into the 21st Century we have gained a far greater understanding of the molecular biology, pathology and other factors that lead to the adult CNS being non-supportive and indeed actively inhibitory to axonal regrowth. On the basis of these recent advances in knowledge, the author outlines possible therapeutic approaches that may enable more effective CNS regeneration to be accomplished in the future.

Key words: *Axonal regeneration, CNS injury, neural repair, neurotrophic factors.*

On various occasions, basing ourselves on precise observations by ourselves and others, we have recorded the radical incapacity of central axons, medullated or non-medullated, young or old, to restore interrupted paths of the white and grey matter. In the spinal cord, under propitious circumstances, one sees from time to time cones of growth connected with axons of the white matter and capable of ramifying and growing across the scar. But in the cerebellum and cerebrum this vigorous, though ineffective, attempt to innervate the cicatricial connective tissue is always lacking.

In the nerves the restoration is a revolutionary work, begun with the utmost rapidity and activity, and apparently stimulated by obstacles. In the centres, on the contrary, the apathy or precarious productive attempts of the first few days are succeeded by the most absolute inactivity.

Ramon y Cajal, 1914[1]

Introduction

That certain injuries of the central nervous system in man failed to recover and could not be effectively treated was recognized as far back as 4500 years ago by the physicians of ancient Egypt,[2,3] and by Hippocrates some 2000 years later.[4] During the late 19th century, the evident lack of any significant regenerative repair within the CNS of man and other adult mammalian species attracted considerable attention from the many eminent neuroscientists of this period, that included Brown-Sequard, Stroebe, Bielschowsky, Marinesco and Ramon y Cajal.[1,5–8] This period of intensive observation culminated

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with the publication of Cajal's classic treatise on the subject "Degeneration and regeneration of the nervous system".[1] Cajal confirmed that the severed ends of CNS axons initially attempt to regenerate with the formation of growth cones similar to those observed in divided peripheral nerves, but that this early outgrowth was not maintained. A scientific giant, he was considerably ahead of his time in that he correctly predicted the existence both of neurohumoral growth factors and the more permissive nature of the Schwann cell environment as being of vital importance in the differing regenerative capacities of the CNS and peripheral nervous system (PNS).

Peripheral nervous system vs central nervous system

Unlike CNS injuries, damage to the adult mammalian peripheral nervous system, provided approximation of cut neural ends is maintained, usually results in effective regrowth of axonal processes and some degree of useful recovery. Distal to injury of a PNS nerve, a succession of changes leading to neural repair takes place as originally described by Waller in 1852, cited by Cajal.[1] The processes that take place during anterograde Wallerian degeneration have subsequently been investigated and described in great detail by a number of investigators.[9–16] Essentially, axons together with their myelin sheaths degenerate, myelin debris being removed by macrophages and Schwann cells, leaving behind largely intact endoneurial tubes that consist of a basal lamina and connective tissue. During the period in which myelin breakdown and removal occurs, Schwann cells proliferate within the endoneurial sheaths forming longitudinally continuous columns comprised of Schwann cells together with their overlapping elongated processes. The proximal axons undergo regenerative sprouting, usually with four to eight new processes emerging from the proximal stump.[9] The growth cones that lead the regenerating axons, grow towards and into the Schwann cell columns which seem to act both as axonal attractors and directional guides for the regenerative repair process.

Comparative anatomy

Unlike the situation that exists in man and other mammalian species, many adult submammalian vertebrates receiving a CNS injury, such as a complete transection of the spinal cord, can undergo successful regeneration. Axons grow across the injury site and beyond to achieve some degree of functional recovery. Spinal cord regenerative repair has been documented in fish, amphibians and some reptiles by numerous investigators from the latter half of the nineteenth century onwards. For a fully comprehensive review of this early work see Clemente.[17] Within such submammalian species there appears to be a far greater degree of cellular plasticity within the CNS. The neural regeneration that is achieved following spinal cord transection appears to be mediated by an interaction between ependymal cells and the exposed overlying mesenchymal cells.[18–21] In the early weeks after spinal injury, ependymal cells around the central canal redistribute and proliferate. These, together with mesenchymal cells derived from connective tissues around the lesion site, bridge the gap between the cord stumps. CNS axonal growth cones appear readily able to cross such tissue, the ependymal tube serving to guide the regenerating axons.[20]

Embryogenesis

During embryonic development, growing CNS nerve fibres successfully negotiate many intervening structures, often travelling considerable distances to reach their target tissues. Whilst to some extent axonal outgrowth may reflect a predetermined 'growth axis' possessed by the neuron itself,[22] it may aid our understanding of abortive CNS regeneration to consider, first, some of the extrinsic factors that are believed to be important in guiding and encouraging neural growth at this earlier time.

Electrical fields

Ariens Kappers[23] developed Strasser's 1892 electrical theory[24] to formulate his concept of neurobiotaxis. A similar electrodynamic theory of neural development was elaborated by Burr.[25] These theories held in common the proposition that dendritic and axonal growth is influenced by electrical fields. Activation of one part of the nervous system, stimulated by another, very clearly plays an important part both in modifying neuronal development and indeed in neuronal survival.[26,27] There is, however, little convincing experimental work to support the hypothesis that differences in electrical potential *per se* have any significant effect upon either the direction of growth or orientation of CNS axons.

Chemotropism

Chemotropic attraction of the growing tips of axons by diffusible factors secreted from target areas was first proposed by Ramon y Cajal in 1892.[28] This concept was elaborated and later refined, forming the basis of the chemoaffinity hypothesis of Sperry, in which growing axons are believed to recognise their topographical targets on the basis of positional markers in the form of gradients of molecules distributed along primary axes.[29] It would be immensely complex and it appears unlikely that chemotropic pathways alone could guide the growth cone of a growing axon all the way from say a parent neuron in the motor cortex to a spinal motorneuron in the lower dorsal spinal cord (see discussion by Novak and Bolz).[30] There seems, however, little doubt that chemotropic attraction, either by released diffusible or by membrane-bound molecules in target and guide tissues, plays an important role both in the final target approach for growing axons in the developing CNS and again later in life for regenerating axons in the PNS.

Mechanical substratum/adhesive molecules

There is considerable evidence that guidance by mechanical and by contact factors can influence the growth and orientation of axonal processes. Harrison[31] and Ramon y Cajal[32] were two early workers who stressed the importance of pre-existing mechanical structures in directing axonal growth. Rakic's studies[33,34] have shown that axons growing in the developing cerebellum, associate preferentially with radially arranged glia. Similarly, neuroblast migration in the early development of the CNS appears to be mechanically guided by an alignment of glial processes. In the developing CNS the extracellular spaces are greater, and they form orientated intercellular channels that may more readily permit axons to pick a way through towards a specified target, than would be the case in the mature CNS.[35,36] Weiss[37,38] demonstrated that axons growing in tissue culture became preferentially orientated, parallel to the direction of tension forces set up within the substrate growth medium. Weiss called this phenomenon contact guidance. Axonal growth, both during development and during regeneration, is accomplished by growth cones located at the tips of elongating axons.[31,39,40] These are motile pathfinding structures and the region at which new membrane is added to accomplish axonal elongation.[41] Adhesive interactions between the growth cone of a growing axon and the substratum within which it is growing appear to be critically important both for directional growth and the extent of axonal elongation, contact guidance and contact inhibition occurring.[42–45] See also further discussion under substrate adhesion/neurite-promoting factors.

Theories on the causes of regeneration failure in mammalian CNS

These can be considered under the broad headings of:

1. Intrinsic inability of CNS neurons to mount a regenerative response.
2. A CNS environment that is non-supportive or actively inhibitory to neural regeneration.

Intrinsic inability of central neurons to mount a regenerative response

Somal reaction. Neurons wholly contained within the CNS are referred to as central neurons, whilst the term peripheral neuron is used to include all neurons whose processes lie wholly or partly in a peripheral nerve. Following transection of the axon of a vertebrate peripheral neuron, its cell body undergoes a series of changes referred to as the retrograde neuronal response. Initially thought to represent a degenerative response, it is now known that it enables the regenerative response to be mounted. Granular endoplasmic reticulum is broken-down and together with free ribosomes is redistributed towards the cell periphery. Nuclear RNA synthesis is increased together with an increase in cytoplasmic protein synthesis.[46,47] Reactive glial changes occur around the axotomized peripheral neurons with proliferation of microglial cells and hypertrophy of astrocytes. How the cell body receives the message that axonal continuity has been interrupted remains uncertain, but it is presumed that the signal is carried back to the soma by retrograde axoplasmic transport.[48,49] Central neurons react to axotomy in a less consistent manner. Initially, some do exhibit a series of cytological changes very like those seen in peripheral neurons. Subsequently, however, they show a progressive decline in levels of both cytoplasmic and nucleolar RNA content.[50,51] Periods of axon outgrowth during normal neural development and also the time of regrowth in axonal regeneration are characterised by the selective expression of certain specific genes by neurons. These genes encode for proteins which are produced in high levels and transported down into growth cones that are responsible for neurite extension.[52] Growth-associated protein GAP-43 is one such protein that accumulates in neuronal growth cones and is presumed to be an essential building-block for axonal elongation.[53,54] Submammalian CNS or PNS injury and also mammalian PNS injury result in a greatly elevated GAP-43 expression, roughly coincident with the initiation of axon regrowth.[52] In contrast, very little elevation in GAP-43 levels takes place after axotomy of adult mammalian CNS neurons.[55] Although an increase in cellular content of RNA and GAP-43 induction following axotomy is the hallmark of those neurons capable of axon regeneration, while the converse holds for non-regenerating neurons, it is unclear whether this is a cause or the result of ineffective neural regeneration.

Evidence of an initial abortive regeneration by many central neurons. The earliest investigators, in contrast to their findings in submammalian species, reported no signs of regeneration following an injury to the mammalian CNS.[17] According to Clemente,[17] Kahler in 1884 concluded that this was because the CNS contained no Schwann cells. The earliest description of the abortive mammalian CNS regenerative response belongs to Stroebe in 1894.[6] He observed that some regenerating nerve fibres did cross the scar tissue of the transected spinal cord but that they failed to achieve any true restitution of spinal cord tissue. Bielschowsky,[7] Cajal[9,56] and others confirmed these findings. Cajal in particular with his superb histological techniques was able to clearly demonstrate after spinal cord section in mammals, that the proximal stumps of large numbers of transected axons sprouted new axonal processes possessing typical terminal growth cones. After approximately a month, however, these regenerating axons atrophied and ultimately degenerated.[9,56] Cajal came to the conclusion that this was not due to an intrinsic inability of CNS neurons to regenerate or the presence of a neuroglial scar, but rather the absence of a trophic and orientating environment similar to that produced in the lesioned PNS by the proliferated cells of Schwann. Similar findings have been found by more recent investigators[57] with the concept now firmly established of an early regenerative effort by the CNS, but that generally the regenerating axons of central neurons appear unable to continue growing across the transection site and beyond.[17]

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Ability of certain mammalian central neurons to regenerate successfully. Within the CNS, certain adrenergic, noradrenergic, dopaminergic and serotonergic neurons seem to be capable of sustaining a more prolonged regenerative growth response.[58–60] After physical or chemical axotomy their axons undergo regenerative sprouting from the cut ends, with the newly formed axons regrowing within the CNS often for considerable distances. Perhaps related to this ability is a similarly seen ability of monoaminergic axons to undergo a more marked degree of collateral sprouting than is seen with other central neurons.

Peripheral nerve 'bridge' experiments. Tello performed one of the earliest recorded (1911) PNS to CNS implantation experiments.[61] A co-worker of Cajal's, his investigation was prompted by a theory of Cajal that a neurohumoral agent, released by Schwann cells, was partly responsible for the successful regeneration seen in the PNS. Tello implanted predegenerated grafts of sciatic nerve into the mammalian cerebrum and after approximately 2 weeks, noted extensive growth of 'central' fibres into the graft, cited by Clemente.[17] Subsequent workers obtained similar findings using peripheral nerve segments grafted into the CNS either in spinal cord, cerebral or optic nerve injury models.[62–64] During the 1970s, Kao[65–67] performed an elegant series of experiments after first developing a delayed microsurgical grafting technique to reduce scarring and necrosis at the PNS graft/spinal CNS interface. Using this technique, sciatic nerve segments inserted between the ends of a transected spinal cord were invaded by regenerating axons that readily bridged the gap between the cord stumps. Myelination of regenerated axons was also observed. Although this and previous authors' studies all showed significant invasion of CNS-implanted peripheral nerve grafts by regenerating axons, it was not possible to demonstrate firmly if these were derived from central neurons, peripheral neurons or autonomic nerve fibres. More recently, however, the application of retrograde neuroanatomical tracing methods by Aguayo and his co-workers has now firmly established that central neurons in the spinal cord, cerebrum, medulla and retina of adult mammals can extend axons for distances equal to the longest CNS fibre pathways in these animals, through such peripheral nerve graft 'bridges'.[68–71] These findings have now clearly refuted the notion that central neurons are intrinsically incapable of mounting any effective regenerative responses after injury.

CNS environment non-supportive/inhibitory to neural regeneration

Evidence for the hypothesis.

Ability of CNS axons to penetrate PNS grafts but not readily be able to re-enter the CNS. As outlined above, the results obtained from the more recent PNS to CNS implantation experiments conclusively demonstrate that the cut axons of central neurons are eminently capable of regenerating over considerable distances when routed away from the CNS along peripheral nerve grafts. When, however, these same regenerating central axons reach the end of the PNS graft and contact the distal CNS-graft junction, they generally either fail to traverse the PNS–CNS interface or if they do successfully re-enter the CNS they at best grow only 1–2 mm.[70,72]

Inability of regenerating PNS axons to penetrate into CNS. Evidence of the generally non-permissive nature of the CNS environment towards axonal regeneration is also provided by studies that involve the dorsal sensory root and its attachment to the spinal cord. Projecting into the dorsal root for some 100–1000 μm is a conical transition zone (TZ) consisting of interlacing islands of central and peripheral nervous tissue.[73,74] When the central branch of a dorsal root is interrupted by crushing, or severance with immediate reanastomosis of its cut ends, the divided axons successfully regenerate through the lesion their growth unimpeded within the columns of reactive Schwann cells and their basal laminae. When, however, these vigorously growing axons encounter the

CNS at the TZ, the majority either stop completely or turn back towards the periphery.[74–77] Similar findings are obtained if a ventral motor root is divided and coapted to the central process of a divided dorsal root. The motor axons regenerate along the dorsal root, but again cease to progress when they encounter CNS tissue at the TZ.[78]

In vitro *experiments. In vitro* studies, in which dissociated peripheral sensory or sympathetic neurons are confronted with explants either of adult rat PNS (sciatic) or CNS (optic) nerves show that these differences in regenerative growth capacity within peripheral or central nervous tissue environments, persist also in tissue culture.[79] In the same cultures, in which up to several hundred axons could be found in the sciatic nerve explants, neurite ingrowth into optic nerves was completely absent.[80] Cryostat-cut sections of the spinal cord, used as an *in vitro* substrate, similarly support only minimal neurite outgrowth as compared with tissues taken from the peripheral nervous system.[81]

Foetal transplants seem exempt. A clear exception to the non-permissive nature of the adult CNS towards axonal growth, is the target-specific and long-distance fibre outgrowth achieved by some transplanted foetal neurons. Studies by Bjorklund and co-workers,[82,83] Tonder,[84] Wictorin,[85] Stromberg[86] and Raisman's group[87] have shown that donor embryonic hippocampal, or septal neurons from more than one species and also human neuroblasts, when transplanted into an adult mammalian CNS environment can form axons which appear able to grow into and innervate either local or distal target fields within a host animal (*vide infra*).

The conclusions that we can draw from all of the above is that with the exception of certain monoaminergic neurons, weakly myelinated systems, and foetal transplants, the adult mammalian CNS presents a microenvironment that is non-supportive or, possibly, actively inhibitory to the regenerative growth repair capacity of both peripheral and central neurons. In contrast, a PNS-type environment seems capable of supporting and directing axonal regeneration, not only by peripheral neurons but by central neurons also.

Factors possibly leading to a non-supportive or inhibitory CNS environment

Relative lack of neuronal growth factors. The discovery of a Nerve Growth Factor (NGF) by Levi-Montalcini, Cohen, Hamburger, and colleagues[88] that caused a dramatic increase in the growth of certain neurons, confirmed the far-sighted prediction of Ramon y Cajal half a century earlier. It was followed subsequently, by the discovery of numerous other growth factors. Neuronal growth factors can exert their effects either through an enhancement of neuronal survival, by promoting axonal growth extension by the neuron or in some instances via both mechanisms. During development, excess numbers of neurons are produced that subsequently are reduced to adult numbers (down by 80% in the case of cholinergic spinal cord motor neurons) by a naturally occurring phenomenon of developmental neuronal death.[89] This takes place around the time when axons reach their target areas and led to the concept that the establishment of successful axon-target contact leads to the protection of that neuron from developmental death. The contacted target tissue producing one or more, necessary neurotrophic factors. Sympathetic and most, if not all, neural crest-derived sensory neurons require NGF for survival during embryonic and early postnatal life.[90] NGF synthesis at this time being primarily by the target tissue for that particular neuron. More recent studies have shown that NGF receptors are also widely present and play a significant role in mammalian CNS development, particularly in the case of cholinergic neurons.[90]

A number of other growth factors with neurotrophic activities *in vitro* have now been identified. These include: brain-derived growth factor (BDGF), ciliary neurotrophic factor (CNTF), epidermal growth factor, acidic fibroblast growth factor (FDGF, basic

FDGF, prototypic nerve growth factor, neurotrophin-3 (NT-3), midkine, pleiotrophin and insulin-like growth factors.[90–93]

Investigations upon neuronal cells growing in culture have emphasized the importance of an additional group of substrate-bound factors present in the extracellular matrix or within the membranes of cells, lacking in any direct neurotrophic activity, but which can promote neurite adhesion and extension. Neurite-promoting factors (NPFs) include laminin, fibronectin, N-Cam, GM_1 ganglioside and contactin (for reviews see Lipton,[94] Sonderegger and Rathjen.)[95] The precise role of these large molecular weight NPFs in directing development and in the regeneration of CNS axons remains yet to be determined, but they are clearly ideal candidates for the selective guidance of growing axons towards their target areas.[96]

In maturity, transection of a peripheral nerve results in large quantities of NGF being produced by the supporting Schwann cells that proliferate within the distal nerve stump. This synthesis requires an interaction with macrophages, which invade the nerve to phagocytose degenerate myelin, and is apparently regulated by the macrophage-derived lymphokine, interleukin 1.[97–100] Transection of peripheral nerves has also been found to lead to a massive increase in local BDNF mRNA levels, with both NGF and BDNF being known to stimulate the regeneration of axons from adult mammalian PNS neurons.[101] Schwann cells furthermore, are a rich source of CNTF-like neurotrophic activity.[102] Isolated CNS astrocytes in culture can also express a variety of neurotrophic factors, (NGF, CNTF, BDNT, NT-3 and FDGF) which are capable of acting upon cultured neurons from both the PNS and the CNS.[103] *In vivo*, however, neuronal growth factors within the normal CNS are predominantly localized to neuronal cell populations.[104,105] CNS injury causes a time-dependent increase in neuronotrophic activity at a lesion site.[105] The appearance of neuronotrophic factors in the mammalian brain after an injury occurs at a slower rate in adults than neonates and to a lower final level of biological activity.[105,106] This latter finding may well account for why CNS implants in adult brains survive and function less well than in newborn animals. A key difference between the peripheral and central nervous system, of relevance to the success or otherwise of regeneration, may be a greater ability of Schwann cells to produce neurotrophic factors in large quantities. Certainly, exogenously supplied neurotrophic factors are able to promote central neuron survival and nerve regrowth following *in vivo* injury.[107–110] The massive increase in BDNF mRNA seen after lesioning peripheral nerves is of special interest in view of the demonstrated ability of peripheral nerve grafts and of isolated Schwann cells to enhance CNS regeneration, particularly in the case of retinal ganglion cells which are supported by BDNF, but not by NGF.[102] Soluble components released by lesioned peripheral nerves can both prevent cell death and induce substantial axonal elongation from isolated retinal ganglion cells in a manner very similar to that seen with exogenous BDNF.[111] A deficient availability of trophic factors for adult central neurons is considered generally by a number of authors to be one of the causes for the relative lack of success of CNS axons in achieving regeneration.[112]

Presence of neurite growth inhibitory factors. Mechanical injury to the adult mammalian CNS always results in the formation, at the lesion site of a dense scar, consisting of elements both of a fibroblast-derived collagenous nature and a glial scar composed of reactive astrocytes together with their cytoplasmic processes.[113–115] Reactive astrocytosis also occurs remote from the site of an injury, in response to CNS demyelination such as following Wallerian degeneration, and also in multiple sclerosis and the degenerative diseases. In contrast, lesions of the foetal and early neonatal mammalian CNS appear to provoke little if any scarring.[116–118] Astrogliosis within the injured CNS includes both a proliferative response, hypertrophy, and an increase in the number of cytoplasmic processes. It is charac-

terised by extensive synthesis of glial fibrillary acidic protein (GFAP) a protein subunit of glial intermediate filaments.[119,120] The accumulation of astrocytes at the lesion margins, results in the formation of an astrocytic boundary or external glial limitans—thicker than that found in the normal CNS glia limitans.[116,120,121] The formation of a glial/connective tissue fibrous scar following trauma is clearly beneficial, in that it re-establishes the integrity of the CNS, sealing it off from the external environment and the inherent risk of infection. The glial/fibroblastic scar has, however, long been considered to represent an impenetrable physical barrier to the regenerative response of CNS axons, largely on the finding that abortively regenerating axons are found within such scar tissue.[9,113] More recent evidence would challenge this view and suggests that the CNS scar does not simply represent a mechanical obstruction to the path of regenerating axons,[122] but instead, inhibits regeneration at the molecular level through cell surface contact-mediated interactions. As reviewed earlier, following division of the central branch of a dorsal root, the interrupted sensory axons successfully negotiate the resultant PNS fibroblast-derived connective-tissue scar, but are halted at the CNS environment of the dorsal root transition zone (DRTZ), where there is a complete absence of fibrous scar tissue, but wherein the contained astrocytes respond to degeneration of the dorsal root afferents by undergoing typical astrogliotic reactive changes.[123] Another challenge to the concept of the CNS scar as being a major physical constraint to axonal regeneration has come from the work of Ann Logan and her colleagues. Their work demonstrates that transforming growth factor B1 (TGF-B1) is one of the first growth factors to be expressed at the site of CNS injury[124] and that it is an important orchestrator of scar production. Neutralization of TGF-B1 activity in a CNS wound can completely prevent the formation of fibrous scar material, yet even with such inhibition, damaged adult axons are still incapable of regenerating across a lesion.[125] By what mechanism then, might reactive astrocytes halt the further growth of regenerating axons in the CNS? In the DRTZ, the growth cones of regenerating axons stop and make stable synaptoid terminals among the processes of reactive astrocytes.[74,77,126] Recently, Liuzzi and Lasek[77,127] have proposed that reactive astrocytes halt the growth of regenerating axons in the mammalian spinal cord by activating an intrinsic physiological stop-pathway that is normally activated in developing axons when axonal growth cones make contact with their appropriate target neurons or peripheral receptors. Other mechanisms whereby reactive astrocytic processes might stop axonal outgrowth have been reviewed by Stensaas.[74] More recently, McKeon and colleagues[128] have demonstrated that there is an increased expression by reactive astrocytes, of tenascin and chondroitin sulphate proteoglycan, cell surface molecules that are inhibitory towards axon growth.

It has been commented upon that some unmyelinated adult CNS axons and neonatal CNS axon systems prior to myelination, but not postmyelination, possess a substantial regenerative capacity.[129] Retinal ganglion cell axons in mammals with unmyelinated retinas, when injured near their cell bodies, are capable in the absence of intact or degenerate myelin of growing for several mm; in contrast, within the myelinated optic nerve environment there is a complete lack of regeneration of these same axons.[130,131] Similarly, unmyelinated hypothalamic neurosecretory fibres within the pituitary stalk can regenerate across the lesion if the pituitary stalk is sectioned.[132,133] These same axons, however, will not regenerate if the cut stalk is approximated to other CNS tissues that contain myelin.[134] Reviewing the above and other experimental findings, Berry[129] proposed the hypothesis that following CNS injury, proteolytic breakdown of mammalian CNS myelin releases axonal growth inhibitory factors (AGIFs) and that these are responsible for the abortive growth response of most axons within the CNS. Schwab and co-workers[90,135-139] in a series of studies, identified in both CNS myelin and oligodendrocyte membranes, two minor

proteins in the molecular weight fractions 33–35 and 250 kDa, respectively, with strong inhibitory effects upon growing neurites (neurite growth inhibitors NI-35 and NI-250). The application of a monoclonal antibody (IN-1) raised against these proteins resulted in a greatly enhanced ability of lesioned corticospinal axons to regrow over longer distances.[139] Pesheva and colleagues have studied the JI-160/JI-180 extracellular matrix glyocoproteins, these are of 160 kDa and 180–200 kDa, respectively. They are expressed relatively late in development by oligodendrocytes and by CNS, but not PNS myelin.[140] The JI-160/JI-180 glycoproteins are implicated in an initially adhesive recognition process between oligodendrocytes and neurons that changes into a repulsive one, as a function of time of interaction between these cells.[141] Whilst much of this type of work has, to date, been focused upon the axonal growth inhibitory properties of CNS myelin it should also be noted that Wallerian degeneration is a necessary prerequisite for adult peripheral nerve regeneration. Adult dorsal root ganglion neurons in vitro only extend neurites on predegenerated and not upon normal peripheral nerves.[142] This latter growth response is independent of the presence of NGF and it raises the possibility that PNS myelin may also be inhibitory towards neurite outgrowth from mature neurons.[142]

Conclusions

It seems certain that CNS oligodendrocytes and myelin, possibly also PNS myelin, possess membrane- or extracellular matrix-associated molecules that inhibit the successful regeneration of adult mammalian CNS and PNS axons. The lack of any significant regrowth of lesioned axons in CNS grey matter (where there is minimal myelin) or in myelin-deficient mutant mammals,[143] together with an inability of regenerating dorsal root sensory axons to cross the DRTZ, highlights also the inhibitory role of reactive astrocytes and a relative lack of neurotrophic molecules as being other important factors for CNS regeneration failure. Whilst regenerating CNS axons are strongly inhibited or halted in their regrowth by such influences, it appears that the initially formed growth cones of transplanted foetal CNS neurons, perhaps lacking in or not yet expressing the appropriately responsive receptors, are exempt from such inhibitory molecular influences.

The future

On the basis of what is presently known, it is possible to envisage three directions for the future development of therapeutic approaches towards enhancing neural regeneration after CNS injury. One approach can be summarized as the 'cocktail strategy'. In this, a balanced mixture of neurotrophic factors, together with antibodies to neurite-growth inhibitory molecules present in CNS-myelin and reactive astrocytes, would be administered to regions of CNS damage and/or seeded along the most important neural tracts leading to and from that region. This could be supplemented by PNS conduit grafts to re-establish links between those centres whose interaction are deemed most important. One experimental example of this type of approach has been the guidance, following optic nerve section, of regenerating retinal axons to the pretectal region with successful re-establishment of a pupillary light constrictive reflex.[144] A second approach is, through the use of microtransplanted embryonic donor cell suspensions, to recreate important innervating centres that have been lost or irreparably damaged. This approach was originally developed for the treatment of Parkinsonism and the neurodegenerative disorders.[145–149] More recently, abundant long fibre growth by axons of embryonic hippocampal donor neurons, microtransplanted into the fimbria of immunosupressed hosts has been achieved, the donor axons successfully making contacts with their appropriate terminal fields.[87] Finally, the most exciting and potentially far-reaching therapeutic approach would modify the regenerative growth cones of disconnected but surviving adult central neurons so that

they behave more like the growth cones of foetal neurons growing *ab initio*, i.e. so that they are no longer susceptible to the neurite-growth inhibitory milieu of the adult mammalian CNS. Clearly, however, before this can even begin to be realized, much more will need to be known about the precise nature of the receptors on the membranes of growth cones that respond either to the growth-inhibitory or growth-promoting, molecular influences that exist within the CNS micro-environment.

In looking to the future it is perhaps fitting to end with the words of one whose research at the beginning of this century provided the foundations for so much that has been discovered since.

> The functional specialization of the brain imposed on the neurones two great lacunae; proliferative inability and irreversibility of intraprotoplasmic differentiation. Once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated.
>
> It is for the science of the future to change, if possible, this harsh decree. Inspired with high ideals, it must work to impede or moderate the gradual decay of neurones, to overcome the almost invincible rigidity of their connections, and to re-establish normal nerve paths, when disease has severed centres that were intimately associated.
>
> Ramon y Cajal, 1914[1]

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EXHIBIT E

Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord

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Transplantation approaches using cellular bridges[1–2], fetal central nervous system cells[3–5], fibroblasts expressing neurotrophin-3 (ref. 6), hybridoma cells expressing inhibitory protein-blocking antibodies[7], or olfactory nerves ensheathing glial cells[8] transplanted into the acutely injured spinal cord have produced axonal regrowth or functional benefits. Transplants of rat or cat fetal spinal cord tissue into the chronically injured cord survive and integrate with the host cord, and may be associated with some functional improvements[9]. In addition, rats transplanted with fetal spinal cord cells have shown improvements in some gait parameters[10], and the delayed transplantation of fetal raphe cells can enhance reflexes[11]. We transplanted neural differentiated mouse embryonic stem cells into a rat spinal cord 9 days after traumatic injury. Histological analysis 2–5 weeks later showed that transplant-derived cells survived and differentiated into astrocytes, oligodendrocytes and neurons, and migrated as far as 8 mm away from the lesion edge. Furthermore, gait analysis demonstrated that transplanted rats showed hindlimb weight support and partial hindlimb coordination not found in 'sham-operated' controls or control rats transplanted with adult mouse neocortical cells.

Neural progenitors isolated from the adult central nervous system differentiate into neurons and glia after transplantation into brain[12], and differentiate into oligodendrocytes and astrocytes after transplantation into spinal cord (F. Gage, personal communication). Another source of undifferentiated cells is embryonic stem (ES) cells, genetically normal immortal cells that have been derived from several species, including mouse and human, and are capable of differentiation into neurons and astrocytes after being transplanted into the brain[13–14]. In our first two series of studies, we induced thoracic spinal cord injury in 22 adult female Long-Evans rats by means of a 10-gram rod 2.5 mm in diameter, falling 25 mm (refs. 15,16). We used ES cell embryoid bodies derived from the D3 line[17] at the 4–/4+ stage (4 days without, then 4 days with retinoic acid) for transplantation. We transplanted partially trypsinized embryoid bodies as cell aggregates into the syrinx that formed 9 days after spinal cord contusion. We handled sham-operated control rats identically, including treatment with cyclosporine, but in place of cell transplantation, they received intra-syrinx injections of culture medium alone ($n = 11$). Beginning on the day of transplantation, all rats received cyclosporine daily to prevent rejection. Hindlimb motor function was assessed using the Basso–Beattie–Bresnahan (BBB) Locomotor Rating Scale[15]. Another group of 11 rats (plus 11 sham-operated control rats) underwent the same transplantation procedure, but using ROSA26 ES cells, a mouse ES cell line containing the *lacZ* transgene and expressing β-galactosidase (β-gal), and rats were killed 2 weeks after transplantation for histology and quantitative cell counting.

Mouse ES cell-derived cells marked genetically (using the ROSA26 line) and pre-labeled *in vitro* with a 24-hour pulse of 10 μM

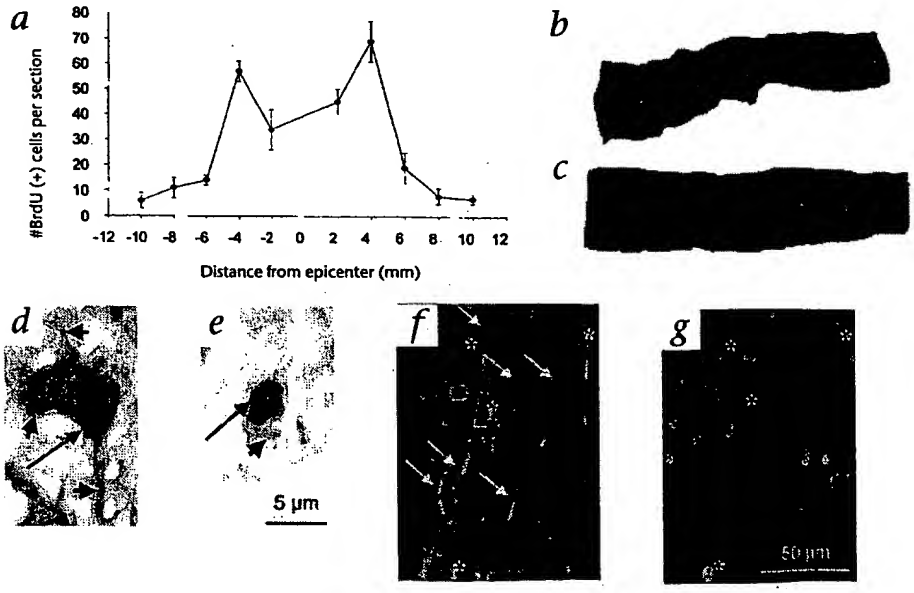

Fig. 1 BrdU labeled ES cell-derived cells 2 weeks after transplantation. *a*, BrdU-labeled nuclei per 1-mm segment in longitudinal sections ($n = 11$ rats; three sections per rat). Data represent mean ± s.e.m. *b* and *c*, Hoechst 33342-labeled sections 42 d after injury, transplanted with vehicle (*b*) or ES cells (*c*) 9 d after injury. *d*, BrdU-positive cell (purple; long arrow) co-labeled with GFAP (brown; short arrows). *e*, BrdU-labeled cell (purple; long arrow) co-labeled with APC CC-1 (brown; short arrow). *f* and *g*, The mouse-specific marker EMA shows processes (arrows) emanating from ES cells (*f*). *, Nuclei in *f* are stained by Hoechst 33342 in *g*.

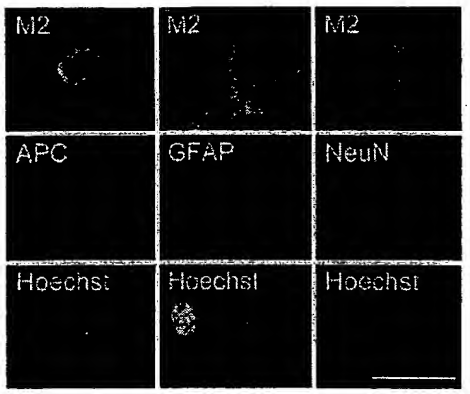

Fig. 2 Transplanted ES cell-derived cells differentiate into oligodendrocytes, astrocytes and neurons. Top row, immunostaining using an antibody against M2 that selectively labels mouse cells, but not rat (identifies transplanted ES cells); middle row, phenotype-specific immunostaining to recognize oligodendrocytes (APC), astrocytes (GFAP) and neurons (NeuN); bottom row, Hoechst 33342 nuclear DNA labeling. Samples are from rats 2 weeks after transplantation; one column represents one cell with three different labels. Scale bar represents 10 μm.

BrdU could be identified *in situ* 14–33 days after being transplanted; identification could also be achieved with the mouse-specific antibodies M2 (ref. 18), EMA (ref. 19) or Thy 1.1/1.2 (data not shown for Thy 1.1/1.2). At 2–5 weeks after transplantation, ES cell-derived cells were found in aggregates or dispersed singly throughout the injury site; furthermore, single cells could be found as far as 8 mm away from the syrinx edge in either the rostral or caudal direction (Fig. 1). In most of the transplanted rats, by 2 weeks after transplantation, ES cell-derived cells filled the space normally occupied by a syrinx in medium-treated rats. By 5 weeks, the density of ES cell-derived cells in this area was reduced and replaced with an extracellular matrix containing fibers positive for Thy 1.1/1.2 labeling. The other mouse-specific markers, M2 and EMA, offered advantages over the genetic and DNA markers (which only mark cell bodies) in that they also labeled ES cell-derived processes, which were abundant in ES cell-transplanted rats, but were not present in sham-operated rats (Fig. 1).

Surviving ES cell-derived cells, labeled with antibodies against mouse-specific markers or BrdU, also labeled with antibodies against markers specific for oligodendrocytes (adenomatous polyposis coli gene product, APC CC-1), astrocytes (glial fibrillary acidic protein, GFAP) and neurons (neuron-specific nuclear protein, NeuN) (Figs. 1 and 2); nuclei could be identified distinctly with Hoechst 33342 staining. Most surviving ES cell-derived cells were oligodendrocytes (43 ± 6% of BrdU-labeled cells were O1-labeled; $n = 11$ rats) and astrocytes (19 ± 4% were GFAP-labeled), but some ES cell-derived neurons (8 ± 5% were NeuN-labeled) were also present in the middle of the cord (Fig. 2). Many of the ES cell-derived oligodendrocytes were also immunoreactive for myelin-basic protein, an integral component of myelin (data not shown). There was no evidence of tumor formation.

Performance in 'open field locomotion' was enhanced by ES cell transplantation (Fig. 3). In contrast to the inability of the sham-operated transplantation group to support weight with their hindlimbs, rats transplanted with ES cells demonstrated partial weight-supported ambulation. A statistical difference in BBB scores was achieved by 2 weeks after transplantation (Fig. 3*a*). After 1 month, there was a difference of two points on the BBB scale between groups: 7.9 ± 0.6, sham-operated (vehicle transplantation); 10.0 ± 0.4, ES cell transplantation. The former score indicates a gait characterized by no hindlimb weight-bearing and no coordinated hindlimb movements, whereas the latter score indicates a gait characterized by partial hindlimb weight-bearing and partial hindlimb coordination.

To assess the possibility that a 'rat-versus-mouse' immune response could contribute to the behavioral benefit, we did a third experimental series. We transplanted rats with 4–/4+ ES cells (ROSA26 line) 9 days after injury and compared them directly with two control groups: culture medium injection and transplantation of adult mouse neocortical cells ($n = 6$ per group). Immunohistologic examination of the spinal cords 5 weeks after transplantation, using antibodies directed against microglia/macrophages (CDIIb) and gamma interferon, showed that all three groups had a similar degree of inflammation (data not shown). Improved locomotor function, as assessed with the BBB locomotor scale (with assignments made using slow-motion video), was again associated only with ES cell transplantation (Fig. 3*b*).

In summary, our study demonstrates that mouse ES cell-derived cells, when transplanted into the spinal cord 9 days after weight-drop injury, survive for at least 5 weeks; migrate at least 8 mm away from the site of transplantation; differentiate into astrocytes, oligodendrocytes and neurons without forming tumors; and produce improved locomotor function. Behavioral recovery similar in magnitude to that shown here has previously only been shown in acute injury models[3–6]. The BBB locomotor score differences between transplanted and control rats were not in the portion of the scale sensitive to forelimb–hindlimb coordination, so this study did not address whether functional connections improved across the lesion site. Further study will be needed to determine the factors responsible for the benefits seen here. One possibility is enhancement of myelination. This is consistent with the rapidity of observed locomotor improvement (2 weeks) and the observation that most ES cell-derived cells were oligodendrocytes, many immunoreactive for myelin basic protein. Transplantation of oligodendrocytes or oligodendrocyte progenitors into demyelinating

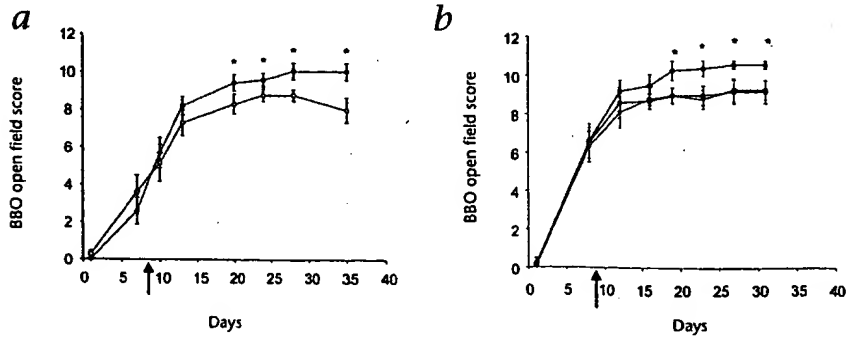

Fig. 3 ES cell-derived cell transplantation improved behavioral recovery. *a*, Change in BBB scores with transplantation, for, ES cell transplant group (●) and vehicle-treated group (○); $n = 11$ per group. *, $P < 0.05$ compared with control at same time point, repeated measures ANOVA with Tukey's test. *b*, Similar experiment to that in *a*, comparing transplantation of ES cells (●), vehicle (○) or adult mouse neocortical cells (◆); $n = 6$ per group. *, $P < 0.05$, ES cell transplantation group compared with both control groups. Data represent mean ± s.e.m. Arrows, transplantation.

chemical lesions can be associated with remyelination and improved axonal conduction[20]. Other possibilities include the reduction of delayed oligodendrocyte death, or the enhancement of host axonal regeneration (for example, by providing a favorable substrate for regrowth, or by producing growth factors).

Methods

Cell culture. D3 (from D.I.Gottlieb) or ROSA26 (from E.J. Robertson) mouse ES cells were maintained and differentiated in culture according to the published 4–/4+ protocol[17]. Undifferentiated ES cells were propagated in the presence of leukemia inhibitory factor (Life Technologies). Cells were cultured as embryoid bodies in the absence of leukemia inhibitory factor for 4 d, then treated for 4 d with retinoic acid (all-*trans*-RA, 500 nM; Sigma). On the ninth day, embryoid bodies were partially trypsinized for 5 min at 37 °C with 0.25% trypsin plus EDTA, and were resuspended in ES cell media[17] before being transplantated.

Spinal cord injury. Impact injury was induced using the weight-drop device developed at New York University ('NYU impact model') as described[15,16]. Adult Long Evans female rats (275 ± 25 g in body weight; Simonsen Lab, Gilroy, California) were anesthetized with pentobarbital (50 mg/kg, intraperitoneal), a laminectomy was done at T9–10 level, and the dorsal surface of the cord was subjected to a weight-drop impact, using a 10-gram weight (2.5 mm diameter) dropped at a height of 25 mm (ref. 16). During surgery, the rectal temperature was maintained at 37.0 ± 0.5 °C by a thermostatically-regulated heating pad (Versa-Therm 2156; Cole-Parmer, Chicago, Illinois), and during recovery, rats were placed overnight in a temperature- and humidity-controlled chamber (Thermocare, Incline Village, Nevada).

Transplantation. BBB scores were obtained the day before transplantation (day 8 after injury), control and experimental groups were matched, and rats were assigned randomly to groups, to ensure that initial locomotor scores were equalized between groups. We chose the weight-drop injury level based on previous experience with the 'NYU impact' model, to produce spontaneous recovery at a BBB score of 8, the most sensitive portion of the scale corresponding to absent weight-supported walking. At 9 d after impact injury, rats received transplants of neural differentiated ES cells (approximately 1 x 10^6), vehicle medium, or 1 x 10^6 adult mouse neocortical cells, by means of a spinal stereotaxic frame, a glass pipette with a tip 100 µm in diameter configured to a 5-µl Hamilton syringe, and a Kopf microstereotaxic injection system (Kopf Model 5000 & 900; Kopf, Tujunga, California).The ES cell or mouse neocortical cell suspension (5 µl) or vehicle medium (5 µl) was injected into the center of the syrinx at the T9 level over a 5-minute period. Three independent experiments, with time-matched controls, were completed in total. The first series was completed for behavioral analysis and late histologic analysis (n = 11 per group): 5 weeks after transplantation; D3 ES line transplantation compared with vehicle medium control. The second series was used to compare early (2 weeks after transplantation) and late (5 weeks after transplantation) histological outcomes (n = 11 per group): ES cell transplantation (ROSA *lac-Z* transgene line) compared with vehicle medium control. In the third series, three groups were compared for behavioral outcome to assess the effects of rat immune reactions to mouse cells (n = 6 per group): neural differentiated ES cell transplantation (ROSA26 *lac-Z* transgene line) compared with mouse neocortical cell transplantation compared with vehicle medium control; survival to 5 weeks after transplantation. All groups received the same daily cyclosporine immunosuppression (10 mg/kg subcutaneously).

Animal care. All surgical interventions and animal care were provided in accordance with the Laboratory Animal Welfare Act, the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, publication number 78–23, revised 1978) and the *Guidelines and Policies for Rodent Survival Surgery* provided by the Animal Studies Committee of Washington University School of Medicine. Bladders were manually expressed three times daily until reflex bladder emptying was established. Cyclosporine (10 mg/kg, subcutaneously) was administered daily to all rats in every group beginning the day of transplantation.

Behavioral testing. Behavioral testing was done weekly using the BBB Locomotor Rating Scale[15] by two individuals 'blinded' to rat treatment status. Behavioral outcomes and examples of specific BBB locomotor scores were recorded using digital video.

Immunochemistry. Primary antibodies used were directed against the following antigens (using the following dilutions): astrocytes (GFAP rabbit polyclonal, 1:4; Incstar, Stillwater, Minnesota); oligodendrocytes (APC CC-1 mIgG$_1$, 1:400; Calbiochem Oncogene Sciences, La Jolla, California); neurons (NeuN mIgG$_1$, 1:500; Chemicon, Temecula, California); mouse EMA rat hybridoma (1:1; from A.L. Pearlman, Washington University; ref. 19); mouse M2 rat hybridoma (from C. Lagenaur, University of Pittsburgh)(ref. 18); BrdU mIgG$_1$ or rat polyclonal (1:400; Boehringer); β-galactosidase mIgG (1:5,000; Promega). Species-specific secondary antibodies (1:200 dilution) were conjugated to Cy3, fluorescein isothiocyanate (Jackson Immunoresearch, West Grove, Pennsylvania) or Alexa 488 (1:200 dilution; Molecular Probes, Eugene, Oregon), and sections were counterstained with Hoechst 33342. Control slides lacking primary or secondary antibodies were analyzed with each series.

Cell quantification. Surviving BrdU-positive ES cells and those double-labeled for markers of differentiated neural cells were counted in three longitudinal sections, centered at the middle of the cord and separated by 200 µm, and results were averaged per rat.

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EXHIBIT F

Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation

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Demyelination contributes to the loss of function consequent to central nervous system (CNS) injury. Enhanced remyelination through transplantation of myelin-producing cells may offer a pragmatic approach to restoring meaningful neurological function. An unlimited source of cells suitable for such transplantation therapy can be derived from embryonic stem (ES) cells, which are both pluripotent and genetically flexible. In this paper we show that oligodendrocyte cultures can be reliably produced from retinoic acid-induced ES cells and that these oligodendrocytes can myelinate axons *in vitro*. Methods were further developed for generating highly enriched cultures of oligodendrocytes through an additional culturing step, producing an intermediate "oligosphere" stage. To test whether ES cells can survive, migrate, and differentiate into mature myelin-producing cells in areas of demyelination in the adult CNS, ES cells were transplanted into the dorsal columns of adult rat spinal cord 3 days after chemical demyelination. In the demyelination site, large numbers of ES cells survived and differentiated primarily into mature oligodendrocytes that were capable of myelinating axons. Furthermore, when oligosphere cells were transplanted into the spinal cords of myelin-deficient *shiverer* (*shi/shi*) mutant mice, the ES cell-derived oligodendrocytes migrated into the host tissue, produced myelin and myelinated host axons. These studies demonstrate the ability of ES cell-derived oligodendrocytes to myelinate axons in culture and to replace lost myelin in the injured adult CNS. Transplantation of ES cells may be a practical approach to treatment of primary and secondary demyelinating diseases in the adult CNS.

Recovery in central nervous system (CNS) disorders is hindered by the limited ability of the vertebrate CNS to regenerate lost cells, replace damaged myelin, and re-establish functional neural connections. In many CNS disorders, including multiple sclerosis, stroke, spinal cord injury, and other trauma, demyelination of intact axons (1–4) is an important factor contributing to loss of function. Previous studies suggest that substantial recovery of function might be achieved through remyelination of otherwise intact axons (5). As a therapeutic modality, functional recovery through remyelination may prove to be a pragmatic approach to regeneration.

Ethical considerations and a lack of a reliable source for undifferentiated pluripotent cells have limited the application of neural transplantation studies in humans. Embryonic stem cells (ES cells) provide a partial solution to these problems because they are genetically normal, pluripotent, capable of indefinite replication (6), and have been derived from several vertebrate species including mice (7, 8) and humans (9, 10, 50). ES cells are also the most flexible stem cell for genetic engineering. Double allele knockouts in single ES cells are possible and such genetic capacities are providing powerful scientific tools (11, 12). Studies examining the regulation of ES cell differentiation into CNS cells are in their infancy. Although ES cells have been shown in culture to differentiate into multiple CNS cell types (13–22), methods for reliably producing oligodendrocytes from retinoic acid-induced ES cells have not been developed. Recently, ES cell-derived oligodendrocytes have been shown to myelinate in the immature CNS (22).

The purpose of the present studies was threefold: (*i*) to develop methods for producing enriched cultures of ES cell-derived oligodendrocytes, (*ii*) to determine whether these cells could myelinate axons *in vitro*, and (*iii*) to determine whether ES cells would survive transplantation into adult spinal cord (both injured and uninjured), migrate, differentiate into oligodendrocytes and myelinate axons. As an injury model, localized chemical demyelination was induced in the dorsal column white matter of rats while sparing passing axons (23–26). Myelin-deficient *shiverer* (*shi/shi*) mutant mice, lacking the gene to produce myelin basic protein (MBP) (27–31), were used as a noninjury model where transplanted oligodendrocytes and newly produced myelin could be identified by immunoreactivity for MBP.

Materials and Methods

Animals and Care. Homozygous adult (*shi/shi*) *shiverer* mice (8–12 weeks old, 17–21 g; 30 mice total) and female Long-Evans rats (8–12 weeks old, 240–270 g; 20 rats total) were obtained from The Jackson Laboratory and Simonsen Laboratories (Gilroy, CA), respectively. Interventions were in accordance with the Laboratory Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, EHEW Pub. No. 78-23, Revised 1978) and the Guidelines and Policies for Rodent Survival Surgery (Animal Studies Committee of Washington University School of Medicine). Anesthesia was induced by ketamine/medetomidine (75:0.5 mg/kg for rats, 75:1 mg/kg for mice; i.p.) and reversed with atipamezole (1.0 mg/kg, s.c.). All animals received (*i*) cyclosporine (10 mg/kg, s.c.) 24 h before transplantation and daily thereafter, (*ii*) antibiotics (enrofloxacin, 2.5 mg/kg, s.c.) before surgery and daily for 3–5 days, and (*iii*) saline (2–5 ml i.p.) and nutritional supplements (Nutri-Cal, EVSCO Pharmaceuticals, Buena, NJ) for 3–5 days after surgery.

ES Cell Culture. ROSA26 (32) or D3 mouse ES cells were used in all experiments, and were differentiated by using the 4−/4+

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Abbreviations: CNS, central nervous system; EB, embryoid body; ES cell, embryonic stem cell; ESIM, embryonic stem cell induction medium; MBP, myelin basic protein; DIV, days *in vitro*; EM, electron microscopy; GFAP, glial fibrillary acidic protein; APC, antigen-presenting cell.

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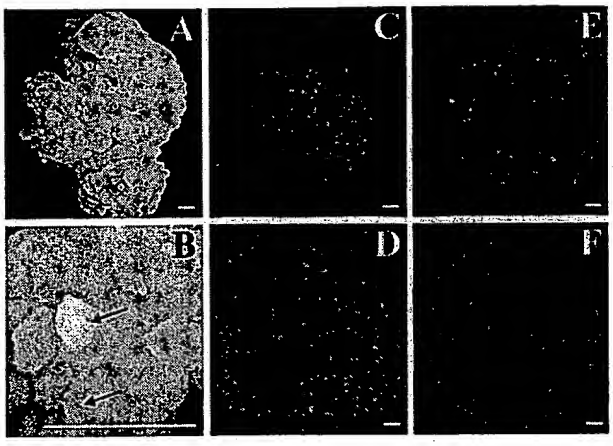

Fig. 1. (*A*) Scanning EM shows 4−/4+ stage EBs, characterized as floating clusters of undifferentiated cells. (*B*) Multiple small processes cover the surfaces of the outer layer cells. Membrane blebbing, consistent with apoptotic cell death, occurs in a fraction of the EB cells (arrows in *B*). Immunoreactivity (green) indicates that the vast majority of EB cells possess immunomarkers for neural precursors (anti-nestin; *F*), whereas few express markers consistent with differentiated neurons (anti-NeuN; *C*), astrocytes (anti-GFAP; *D*), and oligodendrocytes (anti-O1; *E*) (see Table 1). Nuclei are counterstained with Hoechst 33342 (blue). Scale bars = 20 μm.

retinoic acid protocol (15, 33). ES cells were grown in embryonic stem cell induction medium (ESIM, containing DMEM, newborn calf serum, FBS, and nucleosides) for 4 days and ESIM plus retinoic acid (500 nM) for 4 additional days (15, 32). Induced ES cells formed floating clusters of cells, termed embryoid bodies (EBs). After 8 days *in vitro* (DIV), the 4−/4+ stage EBs were partially dissociated (5 min at 37°C, 0.25% trypsin with EDTA) and resuspended in ESIM for transplantation or were further triturated to single cell suspension for plating *in vitro* in modified SATO medium (DMEM with BSA, pyruvate, progesterone, putrescine, thyroxine, triiodothryonine, insulin, transferrin, sodium selenite, amino acids, neurotrophin 3, ciliary neurotrophic factor, and Hepes) (34, 35) with 5% equine serum and 5% FCS.

"Oligosphere" Culture. To produce oligospheres, dissociated 4−/4+ stage EBs were grown in flasks containing modified SATO medium. After 4 DIV, the flasks were gently shaken to suspend loosely adhering cells (primarily oligodendrocytes), while astrocytes remained adhering to the flask. Suspended cells were transferred to new flasks in SATO medium and grown for an additional 2 days. After 6 DIV, oligospheres were partially dissociated (as above) and resuspended in SATO medium for transplantation or completely dissociated for plating in oligosphere-conditioned medium (derived from the shaking step) for *in vitro* studies.

Demyelination. Demyelination was chemically induced in rats by using characterized methods (23–26). After a T10 laminectomy, ethidium bromide (1 μl of 0.1% ethidium bromide in 0.9% saline) or lysophosphatidyl choline (LPC) [lysolecithin (Sigma); 2 μl of 1.0% LPC in 0.9% saline] was injected at 0.5-mm depth in the dorsal column over a 10-min period by using a stereotaxic microinjector (Stoelting) and a 30-μm tip glass pipette attached to a 5-μl Hamilton syringe. Three days later, the demyelinated areas were transplanted with partially dissociated EBs or control medium. Animals were killed 1 and 4 weeks after transplantation (n = 5 each with equal numbers of rats that received vehicle medium sham transplantation).

Preparation of Cells for Transplantation. Prelabeled (see cell tracking methods below) 4−/4+ stage EBs or oligospheres were prepared as described above (33) to produce suspensions of small clusters of cells. Cell density was adjusted to 50,000 viable cells per μl by using a hemocytometer.

Transplantation. Demyelination injury rats received transplants of 125,000 cells from partially dissociated 4−/4+ EBs or medium vehicle. A 50- to 100-μm tip diameter glass pipette was stereotaxically advanced 0.5 mm into the dorsal column white matter. By using a stereotaxic microinjector, 2.5 μl of the ES cell suspension or control vehicle medium was injected at a rate of 0.25 μl/min. The needle was left in place for 5 min and then withdrawn, and the laminectomy site was covered with artificial dura. In the second model, *shiverer* mice were transplanted with 100,000 oligosphere cells or vehicle medium ($n > 6$ each) at the T8 and T10 level (1 μl at two sites 0.35 μm below the dura). Histologic examination was completed at 2 weeks (n = 20) and 4 weeks (n = 10) after transplantation.

Cell Tracking and Immunohistochemistry. Several methods were used to accurately track ES cells after transplantation: (*i*) *lacZ* transgene [ROSA26 line (32)], (*ii*) BrdUrd DNA prelabeling *in vitro*, (*iii*) fluorescent Cell Tracker Orange (Molecular Probes) prelabeling *in vitro*, and (*iv*) mouse-specific Abs. ES cells were pulse labeled with BrdUrd (10 μM; Boehringer Mannheim) for 24 h on the third to fourth day of the 4−/4+ protocol (33, 36). Partially trypsin-dissociated 4−/4+ EBs were incubated with stable fluorescent marker Cell Tracker Orange for 20 min, washed, incubated for another 20 min, and then washed before transplantation. Cell Tracker Orange diffuses into cells and is transformed into a fixable, membrane-impermeant form in the cytoplasm.

Mouse-specific Abs were used to detect the mouse ES cells in the rat demyelination experiments: anti-M2 [labels mouse glia > neurons (37)], and anti-EMA [labels mouse neurons > glia (38)]. Abs used to identify the oligodendrocyte lineage included [see McDonald *et al.* (39) for details of Ab classification]: anti-NG2 for oligodendrocyte progenitors (Chemicon; labels NG2 chondroitin sulfate proteoglycan on the cell surface), anti-O4 for immature oligodendrocytes (Boehringer Mannheim; labels O4 sulfatides on the surface of developing oligodendrocytes), anti-O1 (Boehringer Mannheim; marks O1 sulfatide on mature oligodendrocytes), and anti-adenamatous polyposis coli (APC) (Calbiochem; labels cytoplasmic product of the adenomatous polyposis coli tumor suppressor gene product, found in cell bodies of mature oligodendrocytes) were used to identify mature oligodendrocytes (40). Anti-MBP (Boehringer Mannheim; labels an integral membrane protein in myelin) was used to label terminally mature, myelin-producing oligodendrocytes. Homozygous *shiverer* (*shi/shi*) mice are devoid of MBP because they lack the functional gene. Thus, the presence of MBP^+ myelin in these animals following transplantation provides a useful marker for confident identification of transplanted oligodendrocytes (27, 28). Anti-neuron-specific nuclear antigen (Chemicon; identifies a neuron-specific nuclear protein in postmitotic cells), anti-neuron-specific enolase (Chemicon; reacts with neuron-specific enolase), and anti-β-tubulin III (Sigma; reacts with neuron specific tubulin) were used to identify neurons.

Table 1. Expression of markers of differentiated neural cells and activated caspase 3 in EBs and oligospheres

Cell type	CM1, %	NeuN, %	Nestin, %	GFAP, %	O4, %	O1, %
EB	6 ± 4	6 ± 2	89 ± 2	13 ± 1	38 ± 3	35 ± 5
Oligosphere	10 ± 5	27 ± 4	9 ± 4	6 ± 2	51 ± 7	54 ± 9

Data are given as cell-type percent as indicated by immunoreactivity to phenotypic markers in EBs and oligospheres (mean ± SEM, n = 5 for each).

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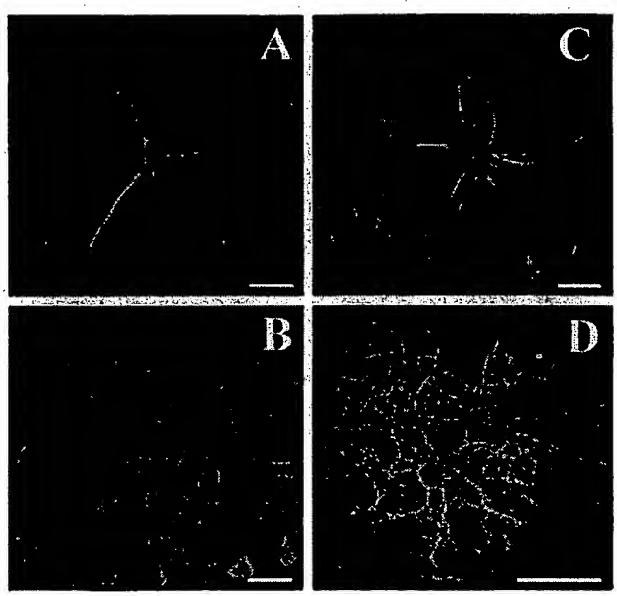

Fig. 2. Mixed cultures of neurons, astrocytes, and oligodendrocytes can be produced by plating dissociated 4−/4+ stage EBs in SATO-defined medium (34, 35) supplemented with 5% FCS and 5% equine serum. Examples of neurons (anti-β-tubulin III; *A*), type-I astrocytes (anti-GFAP; *B*), type-II astrocytes (anti-GFAP; *C*), and mature oligodendrocytes (anti-O1; *D*) are seen in mixed cultures (DIV 9). Scale bars = 20 μm.

Electron Microscopy (EM). EBs and cultures were processed by using standard methods (41). Samples were viewed with a Hitachi S-450 scanning EM operated at 20 KV accelerating voltage and JEOL 100CX transmission EM.

Results and Discussion

ES cells are theoretically capable of differentiating into any cell type, and previous work has shown that they can be neural-induced *in vitro* by using a number of protocols (16, 20–22). Retinoic acid exposure conditions have been developed to restrict differentiation down a neural lineage, and such treatment has been shown to produce cultures containing primarily CNS cells (15). ES cells cultured by using a 4−/4+ retinoic acid protocol (15) have been shown to develop into oligodendrocytes after transplantation into the injured spinal cord (33). This protocol was used as the basis for methods developed to reliably produce both mixed cultures of oligodendrocytes, neurons and astrocytes as well as enriched oligosphere-derived cultures containing predominantly oligodendrocytes.

ES cells grown under the 4−/4+ protocol form floating clusters of cells, termed EBs. Ultrastructural scanning EM examination of EBs revealed that the cells on the surface were covered with extensive microprocesses analogous to the extraembryonic visceral endoderm layer (Fig. 1 *A* and *B*). Immunohistochemical studies of EBs showed limited expression of markers of differentiated neural cells (Fig. 1 *C–F*, Table 1), and the majority were nestin-positive, a marker of neural precursors. Ultrastructural EM evidence suggested that a substantial number of EB cells exhibit features of apoptotic death (Fig. 1*B*). Consistent with these data, chromatin condensation (visible in Hoechst stained nuclei) and immunoreactivity to the activated form of caspase 3 were present in 6 ± 4% (n = 6 EBs) of cells within 4−/4+ stage EBs [Table 1; anti-CM-1, IPO13, Idun Pharmaceuticals, La Jolla, CA (42)].

Plating of dissociated 4−/4+ stage EBs in neurobasal media (Gibco/BRL, catalogue no. 21103-049) produced mixed cultures of neurons, astrocytes, and oligodendrocytes (Fig. 2). Like primary cultures, ES cell-derived type-I astrocytes formed a confluent layer, the other cells grew on top, and neurons grew in small clusters with large bundles of axons radiating outward. Mixed cultures grew best in SATO-defined medium supplemented with serum, and could be maintained for at least 1 month. Inhibition of cell division (10^{-5} M cytosine arabinoside), as used in previous studies of cultured ES cell-derived neurons (15), greatly limited oligodendrocyte viability (27 ± 5 no inhibition vs 0.5 ± 0.2 with inhibition, number of O1-positive cells per ×200 field, n = 4, $P < 0.05$, independent Student's t test).

By using immunohistochemical markers as well as scanning and transmission EM, it was observed that ES cell-derived oligodendrocytes could rapidly and consistently myelinate axons in culture (Fig. 3). Individual oligodendrocytes that simultaneously wrap multiple axons and multiple segments of single axons could be easily identified by using fluorescent Abs directed against components of myelin (O1, MBP). Transmission EM verified that the wrapping observed in the immunocytochemical

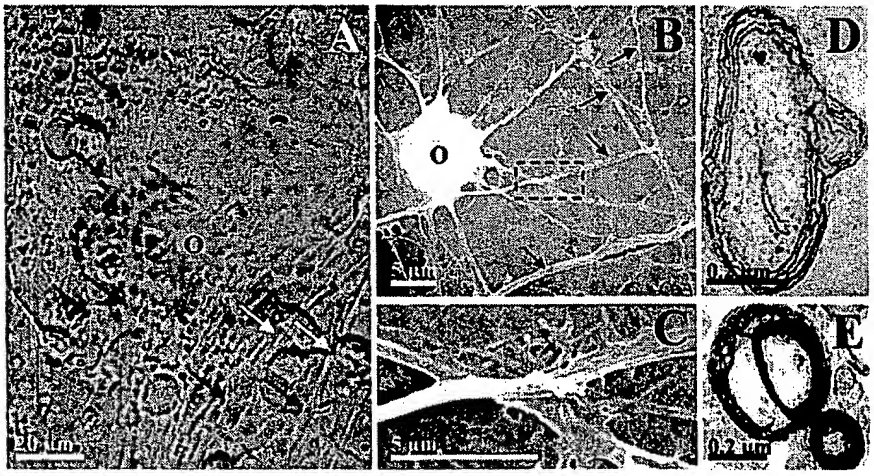

Fig. 3. (*A*) ES cell-derived oligodendrocytes are capable of myelinating multiple axons in culture. O1 immunoreactivity (green) is superimposed on a phase-contrast image in a mixed ES cell-derived neuronal/glial culture (9 DIV). White arrows indicate axons and red arrows indicate O1 immunoreactive wrapped axon segments. (*B*) Scanning EM shows oligodendrocyte (O) and passing axons (black arrows). Higher magnification (box from *B*) demonstrates early axon wrapping (red arrows) by an oligodendrocyte process (*C*), which is similar to early phases of myelination described in studies using video microscopy (49). Transmission EM shows myelin profiles typical of early myelination in 9 DIV culture (*D* and *E*).

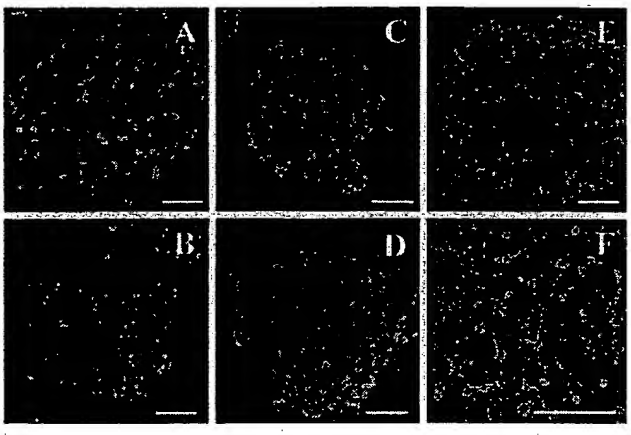

Fig. 4. Oligospheres are floating cell clusters produced from EBs through an additional culture step and yield highly enriched cultures of oligodendrocytes when plated in oligosphere-conditioned medium. Oligospheres are immunoreactive for early markers of neurons (anti-NeuN; *A*), oligodendrocytes (anti-O4; *C*, anti-O1; *D*), neural precursors (anti-nestin; *E*) but few astrocytes (anti-GFAP; *B*) (see Table 1). Oligosphere-derived cultures are enriched for O4 (positive) oligodendrocytes (5 DIV; *F*). Hoechst 33342 (blue). Scale bars = 50 μm.

studies indeed represented myelination. By 9 DIV, evidence of immature axonal myelin profiles with two to five loosely wrapped layers and prominent inner/outer tongue processes were common (Fig. 3*D*). Surprisingly, more mature, highly wrapped (10–15 layers), compact myelin profiles could already be seen by 9 DIV. Previous studies, using primary-derived cultures, suggest that early forms of myelination typically do not appear until 21 DIV, and that development of compact mature myelin profiles typically takes 4–6 weeks. Evidence of early axonal degeneration was also evident in many myelin profiles. This is likely a reflection of the nondepolarizing conditions used in our culture system, which limit neuronal viability past 14 DIV. In the absence of axons, oligodendrocytes formed sheets of myelin, similar to cultures of primary oligodendrocytes (39).

To produce cultures containing primarily oligodendrocytes, an additional culturing step was developed. Dissociated 4−/4+ stage EBs were transferred into flasks containing 5 ml of oligosphere medium, which promotes survival and proliferation of oligodendrocyte progenitors. After 4 DIV, nonadhering cells, consisting primarily of oligodendrocyte precursors, were passed into fresh oligosphere medium at a 1:1 ratio. The few astrocytes generated in this culture adhered to the flask and were not passed. Free-floating spherical cell clusters were formed over 6 days. We termed these clusters oligospheres because later plating produced cultures containing primarily oligodendrocytes. Immunohistochemical studies suggest that oligospheres contain substantial numbers of immature and mature oligodendrocytes, but few astrocytes (Fig. 4, Table 1). In contrast to the high percentage of nestin-positive cells in EBs, only minorities of oligosphere cells were nestin positive. Oligospheres also appeared to contain substantially more cells that immunolabeled for markers of neurons (anti-neuron-specific enolase, NeuN, and neurofilament), although these cells typically did not have processes or ultrastructure suggestive of differentiated neurons. Plating dissociated oligospheres in oligosphere-conditioned medium selected for oligodendrocytes and yielded cultures comprised of 92 ± 7% oligodendrocytes (n = 4). Despite the neuron marker expression in oligosphere cells, very few neurons were observed in any derived cultures.

To evaluate differentiation of ES cells *in vivo*, in the "injured" adult CNS partially dissociated 4−/4+ stage EBs were transplanted into the dorsal column of rat spinal cord 3 days after chemical

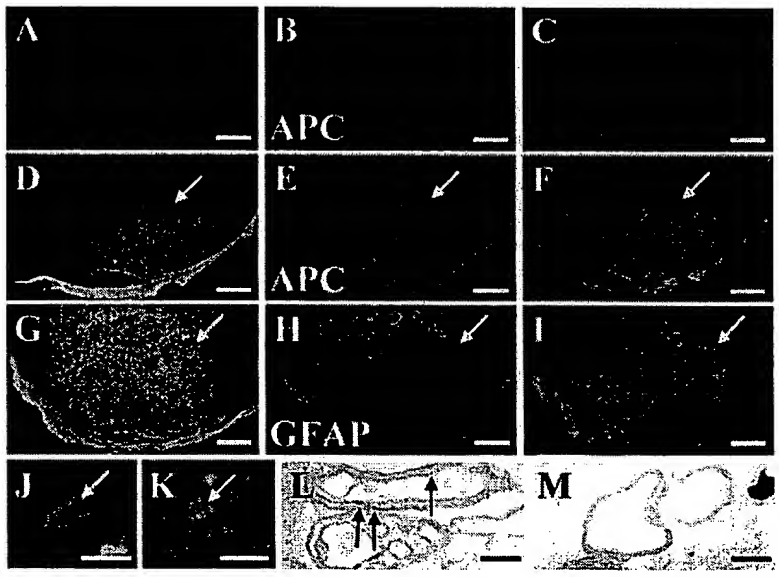

Fig. 5. Transplanted 4−/4+ stage EB cells are capable of differentiating into myelin forming oligodendrocytes in demyelinated adult rat spinal cord. Partial coronal cross-sections (dorsal surface downward) from rats that received sham-transplantation (*A–C*) or ES cell transplantation (*D–F* and *G–I*, two separate animals) demonstrate immunoreactivity for anti-M2 (*A*, *D*, and *G*), anti-APC (*B* and E) and anti-GFAP (*H*). Anti-M2 is a mouse-specific Ab for recognition of transplanted mouse ES cells (37), whereas anti-APC labels oligodendrocytes (40). Increased nuclear density at the site of transplantation is indicated by corresponding Hoechst 33342 staining (arrows; *F* and *I*) compared with control (*C*). ES cell-derived oligodendrocytes primarily occupy the zone of demyelination, whereas there is a corresponding paucity of GFAP immunoreactivity in this area (arrows; *D*, *E*, *G*, and *H*). High magnification shows a native MBP immunoreactive cell (green) from the ventral column distant from the site of transplantation (white arrow, *J*) and a probable transplanted MBP immunoreactive cell from the center of the area of transplant (white arrow, *K*). Transmission EM shows early M2-positive myelin (black arrows indicate DAB precipitate particles associated with M2 immunoreactivity) produced by a transplanted ES cell-derived oligodendrocytes (*L*), and early M2-negative native myelin (*M*) in ultrathin sections without EM staining. Typical EM myelin contrast staining was not done to be able to visualize the diaminobenzidine (DAB) precipitate associated with anti-M2 immunoreactivity. Scale bars: *A–I* = 1 mm; *J* and *K* = 20 μm; *L* and *M* = 0.5 μm.

demyelination. Successful engraftment in the demyelinated region was evident in 9 of 10 rats when examined 1 week after transplantation. This was indicated by immunostaining with anti-mouse-specific Abs (Fig. 5 *A* and *D*), and by an increased cell density demonstrated by Hoechst 33342 labeling in animals receiving transplants. In rats that received a sham vehicle medium transplant, axons of passage were largely spared, as shown previously (23–26), and a paucity of Hoechst 33342 nuclear labeling was present at the site of demyelination. At the lesion site in rats receiving transplants, ES cells differentiated primarily into oligodendrocytes (anti-APC CC-1, anti-MBP; Fig. 5*B*), but not astrocytes [anti-glial fibrillary acidic protein (GFAP)] or neurons (anti-NeuN; data for neurons not shown). A normal low density of host oligodendrocytes (determined by anti-APC, MBP, CNPase, O1) was evident outside the area of demyelination in rats that received transplantation of ES cells or control medium. The host immunoreactive oligodendrocytes are not evident in the low magnification views of Fig. 5 because of the need to prevent over-saturation of high immunoreactivity at the transplantation sites. Enhanced anti-GFAP reactivity was consistently observed at the lesion borders in rats that received both ES cell and vehicle medium transplantation, indicating the association with host reactive astrocytes (Fig. 5*E*). Lack of double labeling for mouse-specific Abs further supported the host origin of the reactive astrocytes (data not shown). Although immature cells can exhibit GFAP immunolabeling, the GFAP labeling here represented primarily astrocytes based on additional morphological characteristics and lack of labeling for other phenotype markers. Histologic evidence of acute graft rejection was present in 1 of 10 rats that received ES cell transplantation.

A second study was performed to assess the potential for ES cell transplants to differentiate into oligodendrocytes and myelinate in the dysmyelinated adult CNS. Dissociated oligospheres were transplanted into the thoracic spinal cord of adult *shiverer* (*shi/shi*) mice, which lack a functional gene for MBP, an essential component of myelin required for compact mature myelin formation. Transplanted cells were tracked by prelabeling the oligospheres with the fluorescent marker Cell Tracker Orange and BrdUrd, or by detecting MBP expressed by transplanted cells. MBP immunoreactivity is always absent in the host *shiverer* mutant CNS. Two weeks after transplantation, ES cell-derived cell tracker orange (positive) (Fig. 6 *A* and *B*) and MBP (positive) (Fig. 6 *C* and *D*) oligodendrocytes were found in white matter. ES cell-derived oligodendrocytes conformed to the organization that oligodendrocytes normally respect in white matter: they would align with host intrafascicular oligodendrocytes and myelinate axons (Fig. 6 *C–G*). Because homozygous *shiverer* mice do not exhibit MBP immunoreactivity (Fig. 6*E*) (27, 28), all MBP immunoreactivity could be attributed to the transplanted ES cell-derived oligodendrocytes. In mice that received oligosphere transplantation, but not in mice that received sham transplantation, MBP immunoreactivity was evident in a radial longitudinal gradient paralleling the white matter tract. Gross MBP immunoreactivity was consistently observed for 0.5 mm surrounding the sites of transplantation, and individual MBP positive cells could be found at sites more distant (2–3 mm) when examined in rats that received transplantation 1 month earlier. Occasionally small central areas of confluent MBP immunoreactivity also colabeled with Abs against extracellular matrix molecules (laminin, fibronectin; data not shown) and were not considered myelin products.

The longitudinal parallel arrays of MBP immunoreactivity, outlining spaces occupied by axons in white matter, are consistent with myelination and are similar to those observed in previous studies of myelination (43, 44) (Fig. 6 *E–G*). Immunocytochemical evidence of myelination was confirmed by transmission EM. Ultrastructurally, *shiverer* mice lack normal myelination; most axons are not myelinated or contain only one to three loose wraps of myelin (Fig. 6*J*). MBP is required for compact myelin formation. Therefore, the presence of multilayered, compact myelin has been used as the gold standard for demonstrating myelination from transplanted oligodendrocytes. In *shiverer* mice that received transplants of oligospheres 1 month earlier, such evidence was present (Fig. 6*K*).

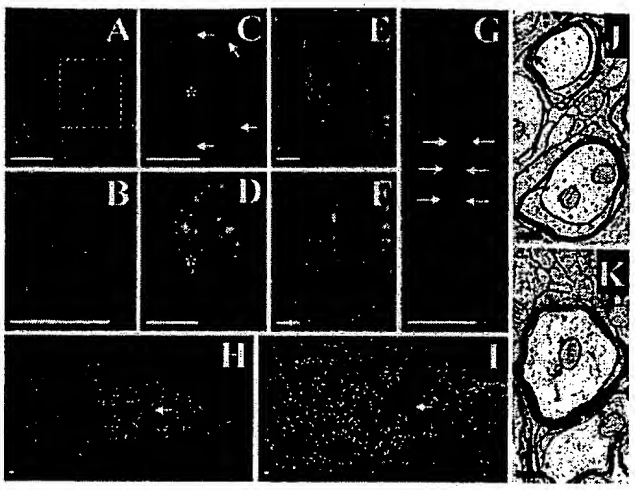

Fig. 6. ES oligosphere-derived cells can migrate and myelinate axons when transplanted into dysmyelinated spinal cords of adult *shiverer* mice, which lack the gene to produce MBP (27–31). Transplanted cells were identified by Cell Tracker Orange epifluorescence (red) or immunoreactivity for MBP (green). Hoechst 33342 (blue). Cell Tracker Orange-labeled cells were found to align with native intrafascicular oligodendrocytes in white matter (*A* and *B*). An ES cell-derived (MBP immunoreactive) oligodendrocyte (asterisk) with longitudinally oriented processes (white arrows) is shown in *C* and *D*. Red arrows mark probable myelination around an adjacent axon (*C*). Little MBP immunoreactivity is present in white matter of a longitudinal spinal cord section from a mouse that received sham transplantation (*E*). A gradient of MBP immunoreactivity is centered on the site of ES cell transplantation (*F*). (*G*) High magnification shows intrafascicular oligodendrocyte nuclei (blue) and MBP immunoreactivity (green) characteristic of axonal myelination (white arrows; refs. 43 and 44) in white matter from a mouse that received ES cell transplantation. The spatial distribution of MBP immunoreactivity, 1 month after ES cell transplantation, is shown at low magnification (*H*) with corresponding Hoechst 33342 counterstaining (*I*). White arrows indicate the center of the transplant. Transmission EM shows four loose wraps of myelin, which represents the maximal number of layers typically seen around axons in control animals (red arrow, *J*), and 9 or greater compact wraps around axons from the area of the transplant (red arrow, *K*). *shiverer* mutant mice lack a functional MBP gene that is required to form mature compact myelin; therefore, the presence of mature compact myelin is a gold standard for transplant oligodendrocyte associated myelin. Scale bars: *A–I* = 10 μm; *J* and *K* = 0.3 μm.

We did not observe any ES cell-derived tumor formation in this study or in our previous studies (33). The pluripotential of ES cells poses a risk for forming normal tissues but in the wrong place (e.g., teratomas). Therefore, it is not surprising that ES cells have produced teratomas under specialized conditions. Because retinoic acid is a strong neural induction signal, we have used it to restrict the cells to a neural lineage to help avoid abnormal tissue formation in the CNS after transplantation. Compared with nonretinoic acid differentiation protocols, the 4−/4+ protocol yields a high percentage of neural cells. However, the culture system is very time sensitive and exposure intervals must be accurate. Transplantation studies of longer duration will be required to adequately assess for the risk of tumor formation. A heartening feature of ES cells is that they can be assessed for genetic normalcy by generating a chimeric animal after implantation into blastocysts.

The present studies demonstrate that ES cells can be used to reliably generate mixed and enriched cultures of oligodendrocytes and that these oligodendrocytes are capable of producing myelin and capable of myelinating axons *in vitro*. In addition, transplanted retinoic acid induced ES cells can: (*i*) preferentially differentiate

into oligodendrocytes in areas of demyelination, and (*ii*) myelinate host axons in the dysmyelinated spinal cord. Our study demonstrates the ability of ES cell-derived oligodendrocytes to myelinate *in vitro* and to show that ES cells survive and myelinate axons in the mature and injured CNS after transplantation. Our findings in the mature CNS are particularly relevant because the most common disorders that are targets for therapeutic strategies of remyelination occur predominantly in adults. In particular, we demonstrate that injured, demyelinated areas of the adult CNS may preferentially stimulate oligodendrocyte differentiation/survival. Since the completion of these studies, another group has reported ES cell-derived oligodendrocyte myelination after transplantation in the immature CNS of myelin-deficient rats (22). A more lengthy induction protocol, not employing retinoic acid, was used that yielded mixed cultures of oligodendrocytes and astrocytes.

Remyelination is a potential mechanism underlying the rapid recovery of locomotor function we observed when dissociated 4−/4+ stage ES cells were transplanted 9 days after moderate spinal cord contusion injury in rats (33). Significant recovery of locomotion was first evident 2 weeks after transplantation. Oligodendrocytes represented the largest identifiable population of differentiated ES cell-derived cells in that study. Transplantation of oligosphere cells in the contusion injury model may provide a useful test of this hypothesis.

The present study suggests that local conditions in the lesioned CNS can select for differentiation or survival of particular types of ES cell-derived neural cells. When ES cells are transplanted into a contusion-injured spinal cord, they differentiate into substantial numbers of oligodendrocytes and astrocytes and <10% neurons (33). In contrast, it is shown here that primary demyelination lesions, sparing passing axons, preferentially support ES cell-derived oligodendrocytes. This observation is compatible with previous demonstrations that CNS isolated progenitors differentiate into different neuronal phenotypes based on their transplantation site in the CNS (45–47).

One important advantage of the use of ES cells for transplantation over primary cells, or other neural progenitors, is their flexibility for genetic engineering. To date, over a dozen double allele knock-ins/knock-outs in single ES cells have been completed (e.g., refs. 11 and 12). This finding provides a powerful scientific and therapeutic tool. ES cells can be genetically modified to produce pro-regenerative factors, such as neurotrophin 3, which has been shown to promote axonal regeneration and myelination (43, 48). These tools will be important for studying the mechanisms that regulate oligodendrocyte differentiation and myelination as well as for developing accurate and rapid methods for quantifying myelin.

Remyelination is a promising and pragmatic approach for repairing the damaged CNS, and strategies are coming within our reach that may offer meaningful recovery of function, such as improved bowel and bladder control, or limb movement. The availability of human ES cells (9, 10, 50) and the possibility of producing autologous ES cells by nuclear transfer provide exciting possibilities for the treatment of many human diseases including those involving demyelination such as multiple sclerosis, Alzheimer's disease, leukodystrophies, and CNS trauma.

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